Extracellular Control of TGF-β Signaling in Glioma

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III. ABBREVIATIONS

ACTR	activin receptor
ADAM	a disintegrin and metalloprotease
ALK	activin receptor-like kinase
AMH	anti-Muellerian hormone
AMHR	anti-Muellerian hormone receptor
AML	acute myeloid leukemia
Ang	angiopoietin
ATRX	α-thalassemia/mental retardation syndrome X-linked
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BRAF	B-Raf proto-oncogene serine/threonine
CDK	cycling-dependent kinase
CDKN2A	cyclin-dependent kinase inhibitor 2A
CHRD	Cys-His rich domain
CNS	central nervous system
CSC	cancer stem cells
D-2-HG	D-2-hydroxyglutarate
ECM	extracellular matrix
EDA	extra-domain A
EDB	extra-domain B
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
EndMT	endothelial-to-mesenchymal transition
ERK	extracellular-signal regulated kinase
FGF	fibroblast growth factor
FN	fibronectin
FOXP3	forkhead box P3

FSA	furin-specific activity
G-CIMP	glioma CpG-island methylator phenotype
GDF	growth differentiation factor
GIC	glioma-initiating cells
Glut	glucose transporter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK3B	glycogen synthase kinase-3β
GTPase	guanosine triphosphate hydrolase
H	histone
HAT	histone acetyl transferases
HCMEC	human cerebral microvascular endothelial cells
HDAC	histone deacetylase
HLA	human leukocyte antigen
ICGC	International Cancer Genome Consortium
IDH	isocitrate dehydrogenase
IGF	insulin-like growth factor
IIICS	type III connecting sequence
IKK	inhibitor of kB kinase
IL	interleukin
iPS cells	induced pluripotent stem cells
JNK	Jun amino-terminal kinase
L1CAM	L1 cell adhesion molecule
LAP	latency associated peptide
LIF	leukemia inhibitory factor
LLC	large latent complex
LTBP	latent TGF-β binding protein
MH	mad homology
MAPK	mitogen-activated protein kinase
MCH	melanin-concentrating hormone
MDM	mouse double minute
MEK	mitogen-activated protein/extracellular signal-regulated kinase kinase
MGMT	0^6-methylguanine-DNA methyltransferase
MMP	matrix metalloprotease

MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
N-cadherin	neural cadherin
NF	neurofibromin
NF-kB	nuclear factor-κB
NGF	nerve growth factor
NK	natural killer
NKG2D	natural killer group 2D
NRP	neuropilin
NSPC	neural stem progenitor cells
OLIG	oligodendrocyte lineage transcription factor
PAI	plasminogen activator inhibitor
PAR	cell polarity regulator partitioning defective
PBMC	peripheral blood mononuclear cells
PCSK	proprotein convertases subtilisin/kexin
PCV	procarbazine, CCNU, vincristin
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
pSMAD	phosphorylated SMAD
PTEN	phosphatase and tensin homolog
PXA	pleomorphic xanthoastrocytoma
Rb	retinoblastoma
R-SMAD	receptor-regulated SMAD
RT	radiotherapy
RT-PCR	real-time polymerase chain reaction
RTK	receptor tyrosine kinase
SBE	SMAD-binding element
SEGA	subependymal giant cell astrocytoma
Shc	Src-homology collagen
SLC	small latent complex
SMURF	SMAD ubiquitylation regulatory factor
SOX	Sex determining region Y-box
SWI/SNF	Switch/sucrose non fermentable

TAK	TGF-β-activated kinase
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-β
TGFβR	transforming growth factor-β receptor
TIMP	tissue inhibitor matrix metalloprotease
TLR	toll-like receptor
TMZ	temozolomide
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
Treg	regulatory T cells
TRH	thyrotropin-releasing hormone
TSP	thrombospondin
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
WHO	World Health Organization
α-KG	α-ketoglutarate
α-sma	α smooth muscle cell actin

1. SUMMARY

Glioblastoma is the most frequent and aggressive intrinsic brain tumor in adults. Transforming growth factor (TGF)-β plays a major role in the malignant phenotype of glioblastoma and has been linked to migration and invasiveness of tumor cells and angiogenesis. Beyond it exerts an immunosuppressive action and sustains the stemness properties and the tumorigenicity of glioma-initiating cells (GIC).

TGF-β is synthesized as a precursor molecule and is stored in an inactive, latent complex in the extracellular matrix (ECM). Different molecules are involved in the processing, secretion and release of the mature form of TGF-β from latent complexes.

The precursor form, pro-TGF-β, is proteolytically processed to the mature form by proprotein convertases subtilisin/kexin (PCSK). The studies performed for this thesis show that furin is unique among the PCSK in being highly expressed in GIC and that furin is the main enzyme responsible for pro-TGF-β1 and pro-TGF-β2 processing in GIC. Notably, pro-TGF-β processing takes place to a greater extent extracellularly, qualifying furin as an extracellular regulator of TGF-β signaling and as a potential therapeutic target to counteract TGF-β in GIC. Also, TGF-β2 controls furin levels and activity in GIC. Indeed, TGF-β2 induces furin expression in an activin-receptor like kinase (ALK)-5-dependent and SMAD-independent manner, involving members of the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) pathway. Specifically, our results uncover a role for ERK1 in regulating basal furin levels and in supporting a self-sustaining loop for high TGF-β activity in GIC. Our data suggest that ERK1 blockade may be a potential therapeutic strategy to inhibit TGF-β in GIC.

The extracellular environment and in particular different components of the ECM such as fibronectin (FN) and fibrillin are involved in the control of TGF-β latency. In glioblastoma, FN and the two alternative FN splice isoforms containing the extra-domain A (EDA), EDA+FN, and extra-domain B (EDB), EDB+FN, are highly up-regulated around tumor vasculature. TGF-β modulates the activation state of endothelial cells, regulating angiogenesis and endothelial cells plasticity. Here we show that TGF-β induces EDA+FN and EDB+FN in endothelial cells. Beyond, FN controls the TGF-β superfamily signaling pathway in endothelial cells, by specifically up-regulating the SMAD1,5 signaling branch via the EDA and EDB domains. This

suggests a positive feedback loop of TGF-β and EDA+FN/EDB+FN in endothelial cells with EDA+FN and EDB+FN maintaining TGF-β superfamily-dependent SMAD1,5 signaling. Further investigation in glioblastoma-derived endothelial cells as well as functional studies may reveal whether EDA+FN and EDB+FN may serve as potential therapeutic targets to combat TGF-β activity in glioblastoma vasculature.

2. ZUSAMMENFASSUNG

Das Glioblastom ist der häufigste bösartige, hirneigene Tumor beim Erwachsenen. Bei der Pathogenese des Glioblastoms spielt der Botenstoff «transforming growth factor» (TGF)-β eine wichtige Rolle, da er das infiltrative Wachstum des Tumors und die Bildung von Blutgefäßen fördert und Tumorstammzellen, die sogenannten Gliom-initiierenden Zellen (GIC), am Leben erhält. Ebenso schützt das von Gliomzellen sezernierte TGF-β den Tumor vor körpereigenen Abwehrmechanismen.

TGF-β wird als Vorläuferprotein gebildet und in der extrazellulären Matrix (ECM) in einem Komplex in einer inaktiven Form gelagert. Verschiedene Moleküle sind an der Prozessierung, Sezernierung und Freisetzung der prozessierten beziehungsweise aktiven Form aus inaktiven Komplexen beteiligt.

Das Vorläuferprotein, pro-TGF-β, wird durch die Enzymfamilie der Proproteinkonvertasen vom subtilisin/kexin-Typ (PCSK) prozessiert. In dieser Arbeit wurde gezeigt, dass Furin unter den PCSK in GIC insofern einzigartig ist, als dass es von GIC stark exprimiert wird und unter den PCSK hauptsächlich für die Prozessierung von pro-TGF-β1 und pro-TGF-β2 verantwortlich ist. Bemerkenswerterweise zeigten die Studien auch, dass die Prozessierung von pro-TGF-β zu einem grossen Teil in der ECM stattfindet. Dies macht Furin zu einem extrazellulären Regulator der TGF-β-Signaltransduktion und einem möglichen therapeutischen Zielprotein, das TGF-β-System in GIC zu hemmen. Im Laufe dieser Arbeit wurde zudem gezeigt, dass TGF-β2 die Furinspiegel und -aktivität in GIC kontrolliert. TGF-β2 induziert die Furinexpression über den TGF-β-Rezeptor «activin like kinase» (ALK)-5, jedoch unabhängig von den Smad-Signaltransduktionsmolekülen. In der Tat erfolgt die TGF-β2-induzierte Furinexpression in einer ALK-5-abhängigen und SMAD-unabhängigen Weise unter Beteiligung von Mitgliedern des «extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK)» Signaltransduktionswegs. Die Untersuchungen der vorliegenden Arbeit identifizierten insbesondere die Isoform ERK1 als Regulator der basalen und TGF-β-induzierten Furinexpresion. Insgesamt illustrieren diese Ergebnisse, dass ERK1 eine wichtige Rolle bei der positiven Regulation dieses selbsterhaltenden Kreislaufs hoher TGF-β-Aktivität spielt und

somit die spezifische ERK1-Blockade eine mögliche Strategie der TGF-β-Inhibition in GIC darstellen könnte.

Das extrazelluläre Milieu und im Besonderen verschiedene Komponenten der ECM wie die Proteine Fibronektin und Fibrillin kontrollieren die TGF-β-Freisetzung aus inaktiven TGF-β-Komplexen. Beim Glioblastom sind Fibronektin (FN) und die beiden alternativen FN-Spleißvarianten, die “extra-domain A” (EDA) und die «extra-domain B» (EDB) enthalten, EDA+FN und EDB+FN, in den Gefäßstrukturen des Tumors sehr stark exprimiert. TGF-β beeinflusst die Aktivierung von Endothelzellen, indem es Angiogenese und Plastizität von Endothelzellen reguliert. In dieser Arbeit wurde gezeigt, dass TGF-β EDA+FN und EDB+FN in Endothelzellen induziert. Darüber hinaus kontrolliert FN den Signaltransduktionsweg der TGF-β-Superfamilie, indem es in den Endothelzellen über die EDA- und EDB-Domänen die SMAD1,5-Signaltransduktion positiv reguliert. Das legt einen positiven, selbsterhaltenden Kreislauf von TGF-β und EDA+FN/EDB+FN in Endothelzellen nahe. Weitere Untersuchungen in direkt von Glioblastomen gewonnenen Endothelzellen sowie funktionelle Studien werden zeigen, ob sich EDA+FN und EDB+FN als therapeutische Ziele im Kampf gegen das Glioblastoma eignen.

3. INTRODUCTION

3.1. GLIOMAS

3.1.1 Classification

Gliomas are the most common primary tumors of the central nervous system (CNS) in the adult. Glioblastoma, representing approximately half of the newly diagnosed gliomas, is the most aggressive brain tumor and has a median patient survival of 14-17 months (Reifenberger *et al.*, 2016).

The cell of origin of gliomas is still controversial. Several studies in animal models suggest that different cell types can generate gliomas including neural stem cells and oligodendroglial progenitor cells (Alcantara Llaguno and Parada, 2016).

According to the most recent World Health Organization (WHO) Classification of Tumors of the CNS published in 2016, gliomas are diagnosed following an integrated approach which combines the histological classification, which was the only criterion used in the previous classification of 2007, with molecular information (Louis *et al.*, 2016; Reifenberger *et al.*, 2016). This approach allows a more precise classification of gliomas with improved prediction of clinical outcome and treatment response.

According to the new classification, the first layer of classification is based on the histological tumor type considering cytological features and expression of lineage-associated proteins of astrocytic or oligodendroglial cells (Alcantara Llaguno and Parada, 2016). The second layer is represented by tumor grading according to the WHO definition taking into account the degree of cell anaplasia. Circumscribed and low-proliferative lesions are assigned grade I. Grade II lesions are low proliferative, but show cytological atypia and are infiltrative. Grade III tumors show hypercellularity, nuclear atypia, and high mitotic activity. Grade IV tumors show characteristics of grade III tumors, with the additional features of microvascular proliferation and necrosis (Louis *et al.*, 2007; Wen and Kesari, 2008). The third layer of classification is based on the molecular characteristics of the tumor. Several genetic and epigenetic alterations have been identified in gliomas (Parsons *et al.*, 2008; Verhaak *et al.*, 2010; Brennan *et al.*, 2013). Large-scale genomic and transcriptomic profiling of gliomas has been realized also in the context of international networks like “The Cancer Genome Atlas (TCGA)” (Cancer Genome

Atlas Research, 2008) and the “International Cancer Genome Consortium” (ICGC) (International Cancer Genome *et al.*, 2010). Some of these molecular alterations are used for the diagnosis of gliomas (Figure 1) or as predictive biomarkers of patient response to therapy and are described in the following.

- IDH. The determination of isocitrate dehydrogenase 1 or 2 (IDH1/2) status represents the first layer of the molecular diagnosis (Figure 1). IDH status is first assessed by immunohistochemistry with an antibody specific for the most common IDH mutation in gliomas (IDH1-R132H). Tumors which are negative by immunohistochemistry undergo molecular testing in order to exclude the presence of other IDH1/2 mutations. IDH mutation is one of the earliest event occurring in gliomas (Suzuki *et al.*, 2015) and identifies biologically different tumors with a different clinical behavior and improved prognosis when compared to IDH-wild-type gliomas (Parsons *et al.*, 2008; Sanson *et al.*, 2009; Yan *et al.*, 2009). Mutated IDH proteins acquire the ability to convert α-ketoglutarate (α-KG) into D-2-hydroxyglutarate (D-2-HG) (Dang *et al.*, 2009). D-2-HG acts as a competitive inhibitor of α-KG-dependent dioxygenases, including some DNA and histone demethylases (Xu *et al.*, 2011). This results in an increase in DNA and histone methylation leading to the hypermethylation of CpG islands and a glioma phenotype known as "glioma CpG-island methylator phenotype" (G-CIMP) (Noushmehr *et al.*, 2010; Liu *et al.*, 2012).

- ATRX. After the definition of the IDH 1/2 status, the molecular diagnosis may proceed with the immunohistochemical assessment of the nuclear retention or loss of α-thalassemia/mental retardation syndrome X-linked (ATRX) transcription factor. ATRX is involved in chromatin remodeling (Liu *et al.*, 2012; Watson *et al.*, 2015).

- 1p/19q. The combined loss of genetic material from the p arm of chromosome 1 and from the q arm of chromosome 19, which is the consequence of an unbalanced t(1; 19) (q10; p10) translocation and known as 1p/19q co-deletion, is assessed in patients with histological diagnosis of diffuse astrocytic or oligodendroglial gliomas with IDH-mutant status and nuclear ATRX retention. The 1p/19q codeletion defines oligodendrogliomas, and is used as a biomarker to predict benefit from upfront radiotherapy (RT) and chemotherapy with procarbazine, CCNU and vincristine

(PCV) compared to RT alone in patients with anaplastic gliomas (Cairncross *et al.*, 2013; van den Bent *et al.*, 2013).

- H3-K27M-mutant. The K27M mutation in the histone H3.3 gene characterizes gliomas developing in midline structures (thalamus, brainstem, and spinal cord) (Louis *et al.*, 2016).

- MGMT. 0^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein. It repairs DNA alkylation induced by alkylating agents like temozolomide (TMZ). Glioblastoma with hypermethylated MGMT promoter show reduced levels of MGMT protein, translating into a reduced ability of DNA repair. The MGMT status is usually homogenous within different tumor areas and is maintained at tumor recurrence. In patients with IDH-wild-type status, MGMT promoter hypermethylation is a predictor of benefit from TMZ treatment and increased survival (Hegi *et al.*, 2005; Wick *et al.*, 2012).

According to the new classification, glioblastomas are divided into two categories based on their IDH status: IDH-wild-type and IDH-mutant glioblastomas. IDH–wild-type glioblastomas encompass ~ 90% of cases, and clinically they correspond to *de novo* glioblastomas with a predominance in patients older than 50 years. IDH-mutant glioblastomas cover the remaining ~ 10% and correspond to glioblastomas arising in younger patients and derived from lower grade diffuse gliomas (Reifenberger *et al.*, 2016).

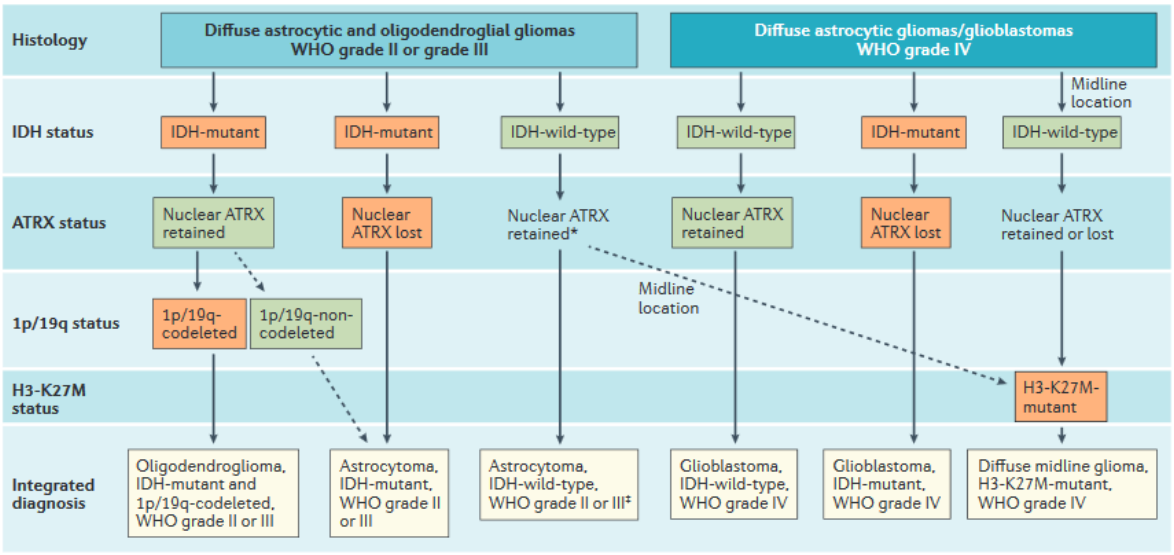

Figure 1. Diffuse glioma diagnosis according to the 2016 World Health Organization Classification of Tumors of the Central Nervous System. The starting point of diffuse glioma diagnosis is the histopathological typing and grading into grade II/III gliomas and grade IV glioma/glioblastoma. The diagnosis than proceeds with the analysis of IDH1/2 status, ATRX nuclear loss, chromosome 1p/19q codeletion and H3-K27M mutations. The integration of the histopathological diagnosis with the molecular information leads to the final diagnosis. IDH1/2, isocitrate dehydrogenase 1 or 2; ATRX, α-thalassemia/mental retardation syndrome X-linked; H3-K27M, histone 3 K27M mutation (adapted from Reifenberger *et al.* 2016).

3.1.2 Molecular pathways altered in glioblastoma

In addition to the genetic and epigenetic alterations described above, many other molecular lesions have been identified in gliomas (Cancer Genome Atlas Research, 2008; Parsons *et al.*, 2008; Brennan *et al.*, 2009; International Cancer Genome *et al.*, 2010; Verhaak *et al.*, 2010). Macroscopic genomic alterations with the loss of chromosome 10 and the gain of chromosome 7 are common in IDH-wild-type glioblastomas (Aldape *et al.*, 2015). Some frequent genomic alterations affect genes encoding for tumor suppressor proteins such as p53 (Watanabe *et al.*, 2001; Cancer Genome Atlas Research, 2008), retinoblastoma (Rb) (Henson *et al.*, 1994; Watanabe *et al.*, 2001; Cancer Genome Atlas Research, 2008) and cyclin-dependent kinase Inhibitor 2A (CDKN2A) (Weber *et al.*, 2007). In particular, TP53 is mutated in 81% of IDH-mutant glioblastomas. In addition to mutations or alterations in the copy number of these genes, the reduced activity of p53 and Rb signaling pathways can also be the consequence of gene amplification events leading to increased levels of p53 and Rb repressors, like cycling-dependent kinase

(CDK)4 and mouse double minute (MDM)2 (Cancer Genome Atlas Research, 2008).

The second group of common alterations in glioblastoma involves receptor tyrosine kinases/RAS/phosphatidylinositol 3-kinase (RTK/RAS/PI3K) signaling pathways (McBride *et al.*, 2010; Verhaak *et al.*, 2010; Snuderl *et al.*, 2011). The most frequently altered RTK in glioblastoma are epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor alpha (PDGFRA) and MET. Around 40% of IDH–wild-type glioblastoma show EGFR amplification. Also, 50% of tumors showing EGFR amplification express a mutated and constitutively active form of EGFR known as EGFRvIII (Frederick *et al.*, 2000; Lee *et al.*, 2006; Hegi *et al.*, 2012; Liu *et al.*, 2015). The EGFRvIII variant has attracted attention since the mutation leading to EGFRvIII generates a novel epitope that may be potentially used as tumor-associated antigen for vaccination therapeutic approaches (Reifenberger *et al.*, 2016). The increase in PDGFR signaling is usually associated with PDGFRA amplification (Verhaak *et al.*, 2010) and promotes proliferation of tumor cells. Amplification of MET has been attributed an important role in gliomagenesis as well (Parsons *et al.*, 2008). Downstream to RTK, mutations in the signaling pathways of RAS and PI3K are common and include: 1) mutations in the gene encoding for PI3K (Suzuki *et al.*, 2015); 2) deletions, mutations or epigenetic alterations affecting the gene encoding for the inhibitor of PI3K pathway, phosphatase and tensin homolog (PTEN) (Wiencke *et al.*, 2007; Huse *et al.*, 2009), which are present in 24% of IDH-wild-type glioblastomas; 3) mutations in the gene of neurofibromin 1 (NF1) (Cancer Genome Atlas Research, 2008; Parsons *et al.*, 2008; Verhaak *et al.*, 2010), a negative regulator of RAS and mammalian target of rapamycin (mTOR) signaling pathways. In some glioblastoma the V600E mutation in B-Raf proto-oncogene serine/threonine kinase (BRAF) is detected (Suzuki *et al.*, 2015). Finally, another frequent genetic alteration affects the telomerase reverse transcriptase (TERT) gene (Arita *et al.*, 2013; Killela *et al.*, 2013; Koelsche *et al.*, 2013). Mutations in the TERT promoter lead to aberrant expression of TERT and are detected in 72% IDH–wild-type and 26% IDH-mutant glioblastomas (Aldape *et al.*, 2015) and present in > 95% of oligodendroglial gliomas (Reifenberger *et al.*, 2016).

3.1.3 Diagnosis and therapy

The standard diagnostic technique for patients with suspected tumor brains is magnetic resonance imaging (MRI). In MRI gliomas appear as heterogeneously enhancing masses surrounded by edema. In glioblastomas, edema is usually more pronounced than in the other gliomas, and the lesions show central necrosis (Wen and Kesari, 2008; Weller *et al.*, 2014).

Surgical tumor resection as feasible is the primary treatment for all gliomas. For non-invasive tumors like pilocytic astrocytoma, pleomorphic xanthoastrocytoma (PXA) and subependymal giant cell astrocytoma (SEGA) surgery is often curative (Reifenberger *et al.*, 2016). For all the other gliomas, additional post-surgery therapies are applied. The type of treatment depends on the tumor type, the expression of specific biomarkers and patient characteristics like age and clinical performance (Weller *et al.*, 2014; Reifenberger *et al.*, 2016). The treatment approaches currently adopted for gliomas therapy are summarized in figure 2. For glioblastoma patients, the standard of care following surgery consists in RT, a total dose of 60 Gy divided into 30 fractions, in combination with daily administration of TMZ. After the combined RT/TMZ treatment, patients receive six cycles of maintenance TMZ (Weller *et al.*, 2014; Reifenberger *et al.*, 2016).

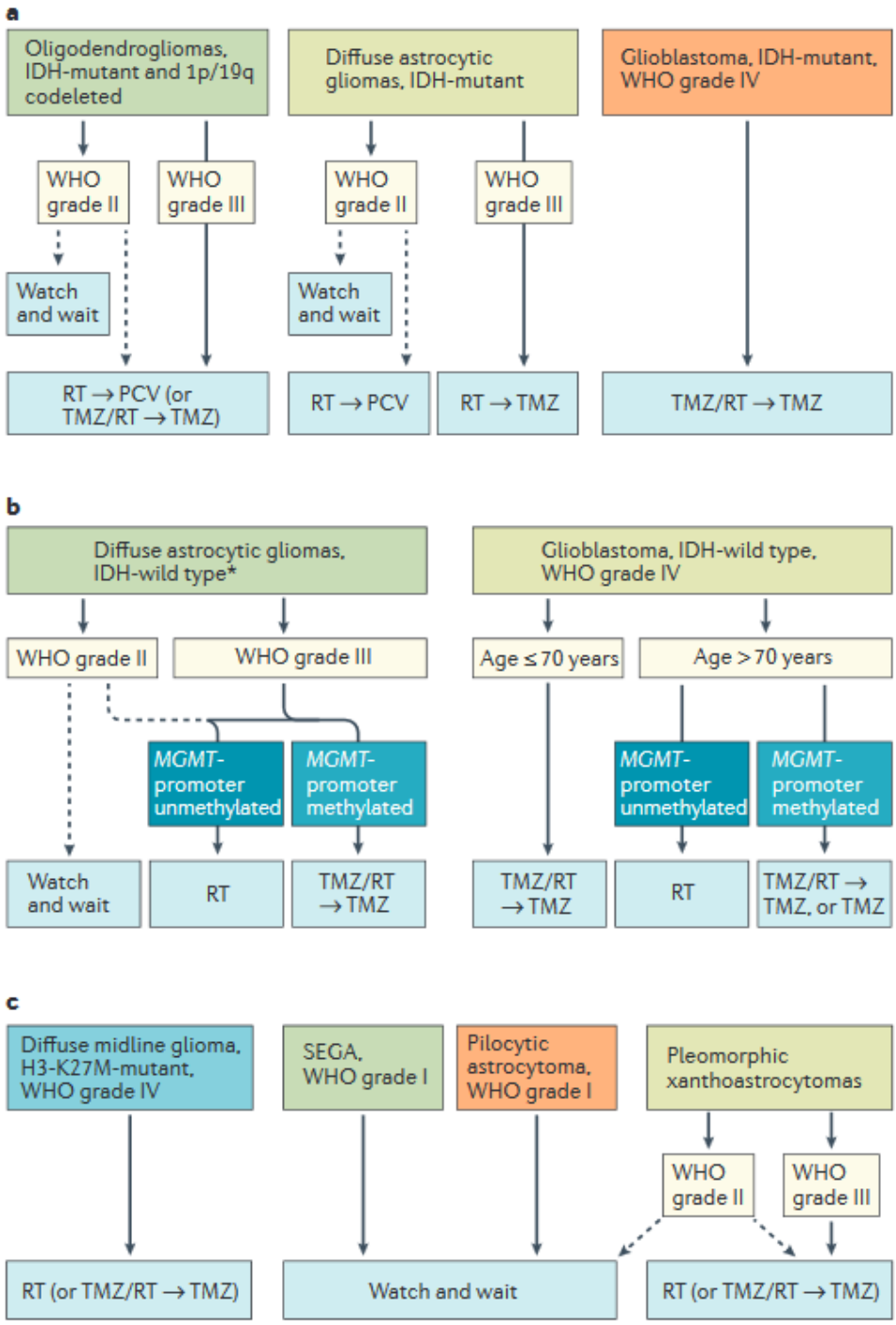

Figure 2. Standard post-surgery treatments in major glioma entities. a. Post-surgery treatment in IDH-mutant adult gliomas. **b.** Post-surgery treatment in IDH–wild-type adult gliomas. **c.** Post-surgery treatment in pediatric gliomas. RT, radiotherapy; PCV, procarbazine, CCNU, vincristine; TMZ, temozolomide; SEGA, subependymal giant-cell astrocytoma (adapted from Reifenberger *et al.* 2016.)

3.2. GLIOMA INITIATING CELLS

3.2.1. Cancer stem cells

Somatic stem cells are defined as rare, self-renewing cells residing in specific niches in adult tissues. Throughout asymmetric division they generate transient amplifying cells, which in turn create lineage-restricted progeny capable of giving rise to terminally differentiated cells (Watt and Hogan, 2000).

According to the cancer stem cell hypothesis tumors contain a small distinct population of tumor cells, the so-called cancer stem cells (CSC), sharing several properties with somatic stem cells including self-renewal, multipotency and asymmetric division and which would be responsible for tumor initiation and recurrence (Reya *et al.*, 2001; Nassar and Blanpain, 2016). The CSC hypothesis thus implies a hierarchical model of tumors with the CSC as a small number of cells at their apex, giving rise to populations of tumor cells with different degrees of differentiation and limited proliferative ability (Reya *et al.*, 2001; Nassar and Blanpain, 2016).

The existence of CSC was first suggested in 1971 by Park and colleagues who described the isolation of mouse myeloma tumor stem cells (Park *et al.*, 1971). The first report on human CSC from Lapidot and colleagues in 1994 showed that a single tumor cell isolated from acute myeloid leukemia (AML) patients was able to initiate AML in mice following transplantation (Lapidot *et al.*, 1994). After extensive documentation in hematological neoplasms, the existence of CSC was also suggested in solid cancers (Reya *et al.*, 2001). The investigation of the potential existence of brain CSC started after the first isolation of neural stem and progenitor cells (NSPC) (Uchida *et al.*, 2000). Indeed, brain CSC, sharing characteristics with NSPC, were first described in anaplastic astrocytoma and recurrent glioblastoma (Ignatova *et al.*, 2002), in medulloblastoma (Hemmati *et al.*, 2003; Singh *et al.*, 2003) pilocytic astrocytoma, ependymoma, ganglioma (Singh *et al.*, 2003) and again glioblastoma (Singh *et al.*, 2003; Galli *et al.*, 2004).

3.2.2. Glioma stem cells/glioma-initiating cells

According to the CSC theory of cancer, CSC would be less sensitive to therapy compared to the bulk tumor and would be responsible for tumor relapse (Reya *et*

al., 2001; Nassar and Blanpain, 2016). Several studies demonstrated the glioma CSC resistance to both chemotherapy and irradiation, pointing towards the necessity to specifically characterize this cell population *in vitro* and *in vivo* (Bao *et al.*, 2006a; Wang *et al.*, 2010a; Venere *et al.*, 2014).

Several methods have been proposed for the enrichment of glioma CSC. One of the most commonly pursued approaches is based on the use of CSC markers. CSC, like somatic stem cells, express stem cell markers. Many glioma CSC stem markers are transcription factors fundamental also for NSPC functions including SRY-box containing gene (SOX)2, Nanog, oligodendrocyte lineage transcription factor (OLIG)2, Myc, Musashi1, Nestin, and BMI1 (Lathia *et al.*, 2015). In addition to these intracellular markers, glioma CSC also express cell surface markers that can be used for their enrichment including CD133, CD15, CD44, L1 cell adhesion molecule (L1CAM) and A2B5 (Lathia *et al.*, 2015)**.** CD133 (Prominin-1), a cholesterol-binding glycoprotein expressed on neural stem cells with unknown functions, was the first cell surface marker proposed and is still the most commonly used marker to enrich glioma stem cells (Singh *et al.*, 2003). Meanwhile diverse studies have indicated that CD133 should be used carefully since only the glycosylated surface CD133 molecule, corresponding to the antigen AC133, is CSC-specific and not CD133 expression *per se*. Indeed CD133 mRNA levels do not correlate with stemness (Kemper *et al.*, 2010). Besides, other studies demonstrated the presence of AC133-negative CSC in gliomas (Beier *et al.*, 2007) or showed that AC133-negative cells can generate AC133-positve cells (Chen *et al.*; Wang *et al.*, 2008), underling the plasticity between the two cell populations.

Overall, the expression of stemness markers is considered not sufficient to define a tumor cell a cancer stem cell, instead a functional validation is needed (Figure 3). Compulsory functional characteristics include sustained self-renewal, persistent proliferation and the capacity, when implanted orthotopically *in vivo*, to generate tumors. Based on their behavior *in vivo* tumor cells with stemness properties are defined according to the following (Lathia *et al.*, 2015):

- Cancer stem cells/cancer stem-like cells (CSC): defined as a self-renewing cell with the ability to give rise to differentiated progeny. Functionally characterized by the capacity of generating a tumor upon secondary transplantation that contains cellular heterogeneity and progeny with varying degrees of self-renewal capacity (Lathia *et al.*, 2015);

- Cancer-initiating cells (glioma-initiating cells (GIC), in the case of glioma): defined as cells with the ability to initiate a tumor upon transplantation. Functionally characterized by the capacity of generating a tumor upon secondary transplantation (Lathia *et al.*, 2015);
- Cancer-propagating cell: defined as cells with the ability to propagate tumor upon transplantation. Functionally characterized by the capacity of propagating a tumor upon serial transplantation (Lathia *et al.*, 2015).

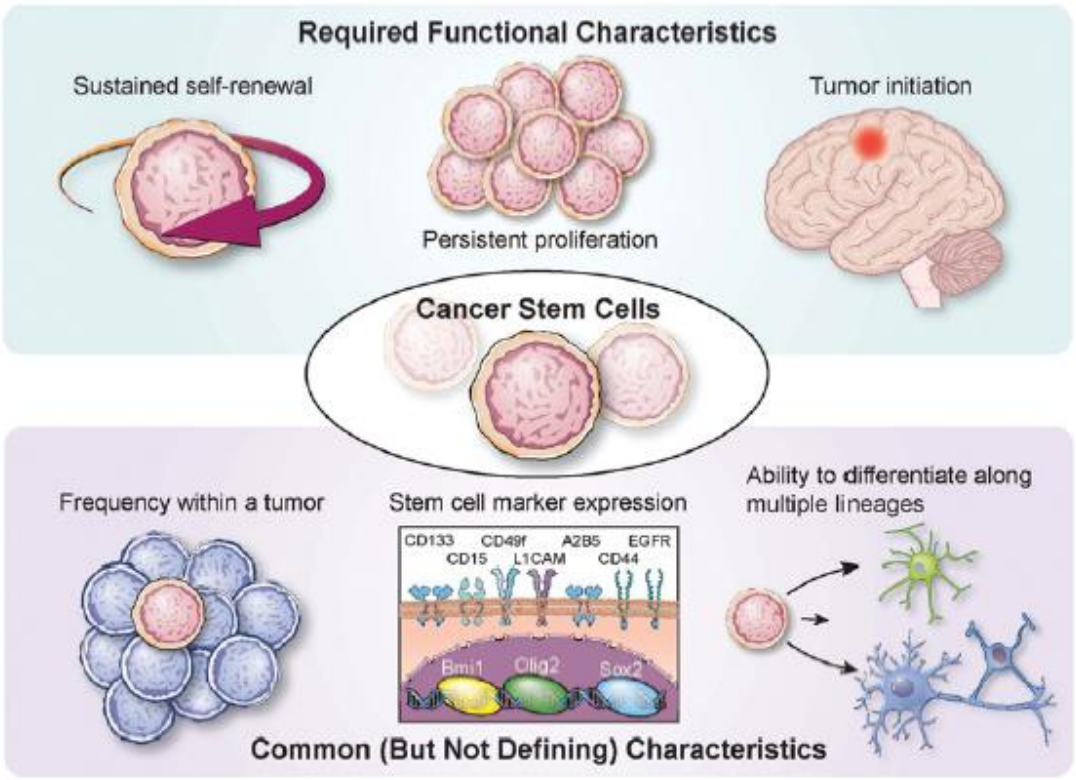

Figure 3. Characteristics of cancer stem cells (CSC) and their functional validation. CSC as somatic stem cells are usually very rare, express stem cell markers and have the capacity of generating differentiated cells of different lineage. Functionally, CSC are defined as cells with self-renewal capacity, characterized by continuous proliferation and by the ability to generate, after orthotopically implantation *in vivo* of a reduced number of cells, tumors recapitulating the tumor of origin (adapted from Lathia *et al*., 2015.).

Alternative methods to the use of stem cell markers for enrichment of glioma CSC include the selection of specific culture conditions which are commonly used for the culture of neural stem cells, i.e. the use of neurobasal serum-free medium containing epidermal growth factor (EGF) and fibroblasts growth factor (FGF) (Lathia *et al.*, 2015). *In vitro* assays such as sphere formation assays may be used

to evaluate proliferation and self-renewal and in case this is done with limiting dilution also to determine the frequency of stem cells. Nonetheless, the golden standard assay to functionally validate CSC is represented by the determination of the ability to generate, after orthotopic implantation of a limited number of cells, tumors *in vivo* morphologically and immunohistochemically recapitulating the tumor of origin and thus showing heterogeneity and cellular hierarchy. Indeed, glioblastoma show high intratumoral heterogeneity and are characterized by the presence of different niches enriched in cells with specific properties, including self-renewal, high proliferation, radio-resistance and adaption to hypoxic conditions (Patel *et al.*, 2014).

3.2.3. GIC and their microenvironment

Somatic stem cells reside in specific niches within tissues with the niche controlling the equilibrium between stem cell quiescence and tissue regeneration (Moore and Lemischka, 2006). In the brain, neural stem cells are located close to blood vessels in two main niches in the subventricular zone and in the subgranular zone (Taupin, 2006). Accordingly, CSC are not casually interspersed in tumors but reside in niches. In glioblastoma two main niches have been identified, i.e. the perivascular (Calabrese *et al.*, 2007) and the hypoxic (Seidel *et al.*, 2010) niches. Concerning the perivascular niche, some studies suggested that CSC can also become an integral part of the vasculature it-self, by trans-differentiating into endothelial cells (Ricci-Vitiani *et al.*, 2010; Wang *et al.*, 2010b) or pericytes (Cheng *et al.*, 2013).

Several soluble factors present in the niche and more generally in the microenvironment of glioblastoma as well as cell–to-cell communications lead to the activation of signaling pathways in CSC that contribute to the maintenance of their undifferentiated state and promote their proliferation. Some of these signaling pathways are also involved in the maintenance of NSPC and may include sonic hedgehog, Notch, nuclear factor-κB (NF-kB), Wnt, PI3K/AKT and nitric oxide signaling (Lathia *et al.*, 2015). Also, glioma CSC survival and self-renewal is influenced by different growth factors and in particular platelet-derived growth factor (PDGF), EGF, and transforming growth factor-β (TGF-β) (Lathia *et al.*, 2015).

The communication between glioma CSC and the cells present in the niche and the microenvironment is bi-directional. This aspect has been investigated mainly with

regard to the glioma CSC-endothelial cells cross-talk. On the one hand, different studies have attested a role for endothelial cells in regulating the stemness of CSC residing in perivascular niches (Lathia *et al.*, 2015). Moreover, a role for the perivascular niche in protecting CSC after radiation treatment has also been suggested (Bao *et al.*, 2006a). On the other hand, glioma CSC express elevated levels of vascular endothelial growth factor (VEGF) (Bao *et al.*, 2006b) and can induce endothelial cells to produce VEGF and brain-derived neurotrophic factor (BDNF) (Li *et al.*, 2006) promoting tumor angiogenesis. In the perivascular niche, glioma CSC come in contact also with cells belonging to the immune system. Immunosuppressive properties have been attributed to glioma CSC, dependent on both cell-cell contact and on the release of soluble factors. Glioma CSC can induce regulatory T (Treg) cells and on the other side inhibit cytotoxic T-lymphocyte proliferation and activation and induce their apoptosis (Di Tomaso *et al.*, 2010; Wei *et al.*, 2010). Also, by releasing interleukin (IL)-10 and TGF-β, glioma CSC can promote the polarization of tumor-associated macrophages towards a pro-tumorigenic phenotype (Wu *et al.*, 2010).

Glioma CSC show high ability to adapt to stressful microenvironmental conditions, including hypoxia and restriction of nutrients. Different studies illustrated the central role played by both metabolic and epigenetic reprogramming in this context. For example, it has been shown that glioma CSC express the high glucose affinity neuronal glucose transporter (Glut)3 to preferentially take up glucose and resist to nutrition restriction (Flavahan *et al.*, 2013). In another study, it has been demonstrated that glioblastoma CSC, through epigenetic reprograming, acquire an increased capacity to take up iron from the environment (Schonberg *et al.*, 2015). Recently, it has been demonstrated how, by chromatin remodeling, glioma CSC enter, in a reversible manner, in a slow-cycling state that allows them to resist to treatment with RTK inhibitors (Liau *et al.*, 2017).

3.3. TRANSFORMING GROWTH FACTOR (TGF)-β

3.3.1. TGF-β superfamily

TGF-β is the prototype of an ancient superfamily of growth and differentiation factors, emerging with the first TGF–β-like growth factor during the early evolution of the deuterostomes (Burt and Law, 1994; Herpin *et al.*, 2004; Robertson and Rifkin, 2013). Members of the TGF-β superfamily have been identified in extremely simple metazoans like sponges (Adamska *et al.*, 2007) and ctenophores (Pang *et al.*, 2011). Evolutionary studies based on sequences belonging to a broad range of species revealed that TGF-β superfamily ligands as well as their receptors and downstream mediators are highly conserved during evolution and led to the identification of several subfamilies (Burt and Law, 1994; Robertson and Rifkin, 2013). Based on structure similarities TGF-β superfamily ligands can be divided into TGF-β, bone morphogenetic proteins (BMP) and activins/inhibins. Concerning the three TGF-β isoforms TGF-β1, TGF-β2 and TGF-β3, TGF-β2 and TGF-β3 cluster to a separate sub-tree when compared to TGF-β1, suggesting that these two isoforms emerged as a consequence of a second gene duplication event occurring later during evolution (Robertson and Rifkin, 2013). In human the TGF-β superfamily consists of 30 members identified so far including the three TGF-β isoforms, ten BMP, eleven growth differentiation factors (GDF), four activin β-chains/inhibin, the anti-Muellerian hormone (AMH) and nodal (Figure 4).

TGF-β superfamily members play fundamental roles in embryogenesis and tissue homeostasis (Wu and Hill, 2009). Concerning the three TGF-β isoforms, knockout mice revealed their roles during embryogenesis. TGF-β1 knockout mice show defects in hematopoiesis and vasculogenesis and die few weeks after birth because of severe inflammation affecting different organs (Kulkarni *et al.*, 1995). Around 60% of TGF-β2 knockout mice die during or soon after birth because of defects affecting several organs and tissues and mainly the heart, the skeleton and the kidney (Sanford *et al.*, 1997). TGF-β3 knockout mice die within few hours after birth mainly because of defective lung development and cleft palate (Kaartinen *et al.*, 1995). The different phenotypes of the three types of knockout mice suggest that, even if TGF-β isoforms show similar effects *in vitro*, they have non redundant functions *in vivo*, which are only in part associated to their different spatial-temporal expression pattern (Robertson and Rifkin, 2013). In addition to the fundamental role played by

TGF-β in development TGF-β signaling is important for the maintenance of tissue homeostasis and the deregulation of its activity is associated with different pathological conditions (Blobe *et al.*, 2000) including inflammation (Han *et al.*, 2012), fibrosis (Meng *et al.*, 2016) and cancers (Massague, 2008).

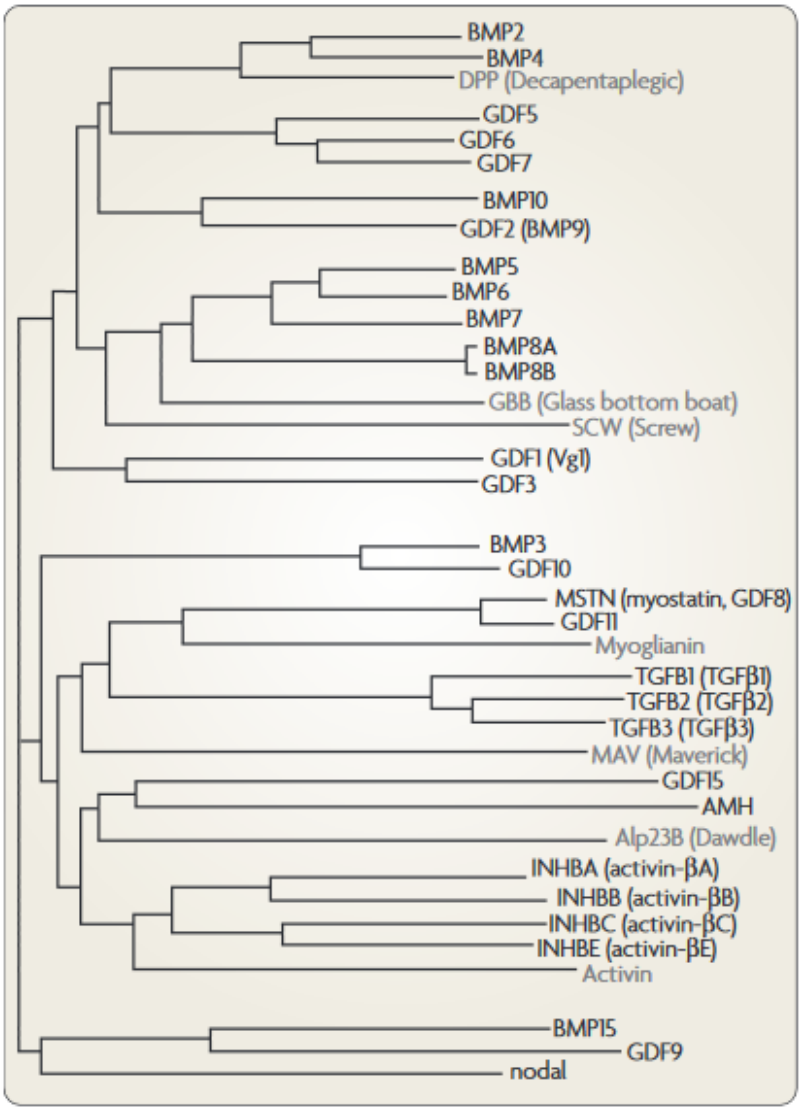

Figure 4. Phylogenetic tree of TGF-β superfamily ligands. Phylogenetic tree of TGF-β superfamily ligands obtained by protein alignment in human and *Drosophila melanogaster*. Human sequences are in black; *Drosophila melanogaster* sequences are in grey (adapted from Schmierer and Hill 2007).

3.3.2. TGF-β processing, secretion, and activation

TGF-β synthesis, processing, secretion, and activation is a multistep process which involves different enzymes/regulatory molecules (Figure 5).

TGF-β is synthesized as a pre-pro-protein, including a signal peptide, an amino-terminal pro-domain and the carboxy-terminal TGF-β family polypeptide (Figure 5a, 1). After removal of the signal peptide, pro-TGF-β covalently homodimerizes in the endoplasmic reticulum (Figure 5a, 2). In the trans-Golgi network TGF-β is then processed by endoproteases belonging to the family of PSKC (Constam, 2014). The two products of this proteolytic process, i.e. a covalent dimer of mature TGF-β and a covalent dimer of the pro-domain, known as latency associated peptide (LAP), form a non-covalent complex known as the small latent complex (SLC) (Figure 5a, 3). The SLC usually forms an inter-molecular disulfide bond with a single molecule belonging to the family of the latent TGF-β binding proteins (LTBP) (Figure 5a, 4) (Oklu *et al.*, 2011). Binding to LTBP favors pro-TGF-β folding and, at a later step, promotes TGF-β secretion. The SLC and LTBP form the so-called large latent complex (LLC). The LLC is released and accumulated in the extracellular matrix (ECM), where it interacts with ECM molecules through LTBP (Figure 5a, 4) (Robertson and Rifkin, 2013; Robertson *et al.*, 2015). Specifically, the amino-terminal regions of LTBP 1,2 and 4 interact with fibronectin and the carboxy-terminus of LTBP 1,2 and 4 interacts with fibrillin (Robertson and Rifkin, 2013; Robertson *et al.*, 2015). In this ECM-associated format mature TGF-β is inactive. To be active it must be released from its latent ECM-associated form. The importance of the ECM in controlling the activation state of TGF-β is exemplified by pathological conditions such as the Marfan syndrome, where a mutation in the fibrillin gene is associated to an excessive activation of TGF-β (Dietz *et al.*, 2005). Besides, mice lacking the fibronectin isoform containing the extra-domain A show reduced TGF-β1 activation (Muro *et al.*, 2008; White *et al.*, 2008).

Several mechanisms allowing the release of TGF-β from the latent ECM-associated complex have been described, and these involve integrins (Wipff and Hinz, 2008), proteases and physicochemical factors (Figure 5b) (Robertson and Rifkin, 2013). The integrins αvβ6 and αvβ8 bind to the LAP and are involved in TGF-β release from the ECM via two different mechanisms. The interaction between integrin αvβ6 and LTBP allows the transmission of traction forces from the cell surface and the ECM to the LLC leading to the release of active TGF-β (Annes *et al.*, 2002; Annes *et al.*, 2004; Fontana *et al.*, 2005). Integrins such as αvβ8 recruit matrix metalloproteases (MMP) on LLC promoting the cleavage of the LAP and the release of active TGF-β (Mu *et al.*, 2002). Concerning proteases, plasmin is the most studied

enzyme involved in TGF-β activation (Lyons *et al.*, 1990). Other proteases include the metalloproteases MMP2 and MMP9 (Yu and Stamenkovic, 2000) and BMP1 (Ge and Greenspan, 2006), kallikreins, calpain (Abe *et al.*, 1998) and cathepsin-D (Jenkins, 2008). Also, de-glycosidase may facilitate TGF-β activation by deglycosylating the LAP and favoring the consequent proteolytic cleavage of the LAP inducing the release of active TGF-β (Miyazono and Heldin, 1989). Other proteins involved in TGF-β activation include thrombospondin 1 (TSP1), F-spondin/spondin-1 and neuropilin (NRP) (Robertson and Rifkin, 2013). Finally, physicochemical factors such as UVB and ionizing radiation, reactive oxygen species, heat and pH extremes can activate TGF-β (Robertson and Rifkin, 2013).

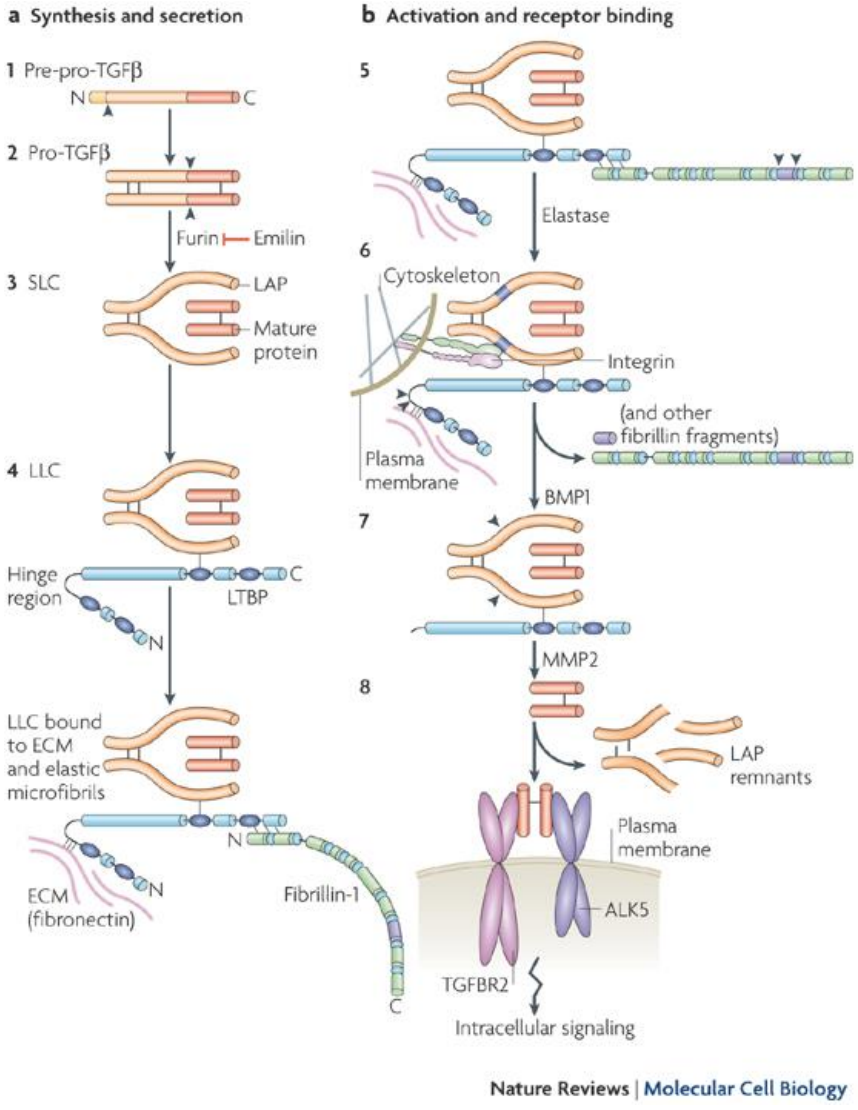

Figure 5. Pictogram of TGF-β synthesis, secretion and activation. The depicted molecules and steps are described in the text. Once released mature TGF-β is active and can bind to TGF-β

receptors (adapted from ten Dijke P. & Arthur E. M. Extracellular control of TGF-β signaling in vascular development and disease. Nature Reviews Molecular Cell Biology 2007, 8: 857-869).

3.3.3. TGF-β signaling

TGF-β superfamily ligands signal to the cell through binding to transmembrane receptors belonging to two functional classes, the so called type I and type II receptors. In human, seven type I and five type II receptors have been identified, i.e. type I/activin-receptor like kinase (ALK) 1-7 and the type II receptors TGFβRII, ACTRII, ACTRIIB, BMPRII and AMHRII (Schmierer and Hill, 2007). TGF-β binds mainly to the type I receptor ALK-5 and, with lower affinity, to the receptor ALK-1 and exclusively to the type II receptor TGFβRII. Both type I and type II receptors are Ser/Thr protein kinases, being the only transmembrane Ser/Thr kinases described so far. Ligand binding leads to the assembly of the tetrameric TGFβR complex made of a dimer of type I receptors and a dimer of type II receptors (Figure 6). Upon ligand binding, type II receptors phosphorylate type I receptors in their Gly-Ser-rich juxtamembrane domain. The phosphorylated Gly-Ser-rich domain recruits the receptor-regulated SMAD proteins (R-SMAD) SMAD1,2,3,5 and 8, which are in turn phosphorylated by type I receptors (Schmierer and Hill, 2007). The receptors ALK-4, ALK-5 and ALK-7 bind to and phosphorylate the R-SMAD2 and 3 and the receptors ALK-1, ALK-3 and ALK-6 bind to and phosphorylate R-SMAD1,5 and 8 (Schmierer and Hill, 2007). Since the main type I receptor of TGF-β is ALK-5, TGF-β signaling mainly results in the phosphorylation of SMAD2 and 3. Nonetheless, in endothelial cells expressing both ALK-5 and the endothelial-specific receptor ALK-1, TGF-β can recruit both the ALK-5 and ALK-1 receptors in one complex and activate both the SMAD2,3 and SMAD1,5,8 branches (Goumans *et al.*, 2003; Byfield and Roberts, 2004).

Upon phosphorylation R-SMAD dimerize and bind to the common mediator SMAD4. In their inactive, dephosphorylated state, R-SMAD shuffle between the nucleus and the cytoplasm. Upon phosphorylation, dimerization and complex formation with SMAD4, R-SMAD/SMAD4 complexes accumulate in the nucleus to interact with transcription factors, co-activators, and co-repressors and bind to DNA. Thus, SMAD complexes regulate gene expression and also regulate chromatin-remodeling and histone modifications by recruiting chromatin-remodeling and

histone-modifying enzymes (Schmierer and Hill, 2007). SMAD proteins consist of two conserved domains, known as mad homology (MH) domains 1 and 2, connected by a linker region. The MH1 domain is a DNA-harping binding domain that allows the direct interaction of SMAD with the DNA. DNA binding occurs at the SBE (SMAD-binding element) DNA motif (Schmierer and Hill, 2007). In the case of SMAD2, the MH1 domain contains a small insert that does not allow SMAD2 to interact directly with the DNA, thus SMAD2 binding to DNA requires its interaction with other DNA-binding proteins. The MH2 domain is responsible for inter-SMAD interaction and SMAD binding to receptors and transcription factors/regulators (Schmierer and Hill, 2007). The linker region can be phosphorylated by several kinases, including MAPK, glycogen synthase kinase-3β (GSK3B) and CDK with regulatory, both positive and negative, functions on SMAD activity (Schmierer and Hill, 2007). The activity of R-SMAD/SMAD4 complexes can be directly regulated by two additional SMAD proteins, SMAD6 and SMAD7, which have inhibitory functions (Schmierer and Hill, 2007).

In addition to the SMAD-dependent TGF-β signaling, which is also called canonical TGF-β signaling, other signaling pathways, known in complex as non-canonical/SMAD-independent signaling pathways have been described for the TGF-β superfamily (Figure 6) (Massague, 2012; Heldin and Moustakas, 2016; Zhang, 2017).

The branches of the non-canonical signaling pathway are activated by diverse mechanisms. Some non-canonical signaling pathways are activated by type II receptors, others by type I receptors. The type II receptor TGFβRII is able to directly phosphorylate and activate the cell polarity regulator partitioning defective 6 (PAR6) which then recruits SMAD ubiquitylation regulatory factor (SMURF)1 and target RHOA guanosine triphosphate hydrolase (GTPase) at tight junctions consequently leading to the dissolution of tight junctions and polarized migration. This process is triggered by TGF-β in cells undergoing epithelial-to-mesenchymal transition (EMT) (Massague, 2012). Type I receptors can activate several non-SMAD signaling pathways, i.e. the p38, Jun amino-terminal kinase (JNK), inhibitor of kB kinase (IKK), mTOR, PI3K, ERK and RHO signaling pathways. Tumor necrosis factor receptor-associated factor (TRAF)6 and SMAD7 directly bind to the receptor I leading to the activation of the TGF-β-activated kinase (TAK)1 which in turn phosphorylates and activates p38, JNK and IKK (Massague, 2012; Heldin and Moustakas, 2016; Zhang,

2017). Phosphorylated TGFβRI first recruits the adaptor protein Src-homology collagen (Shc) with the consequent recruitment of the adaptor protein Grb2 in complex with nucleotide exchange protein Sos1 and activation of RAS, which in turn, activates the ERK1/2 pathway (Heldin & Moustakas 2016). The molecular mechanism responsible for the activation of other signaling pathways, i.e. mTORC, PI3K and RHO is not known (Massague, 2012).

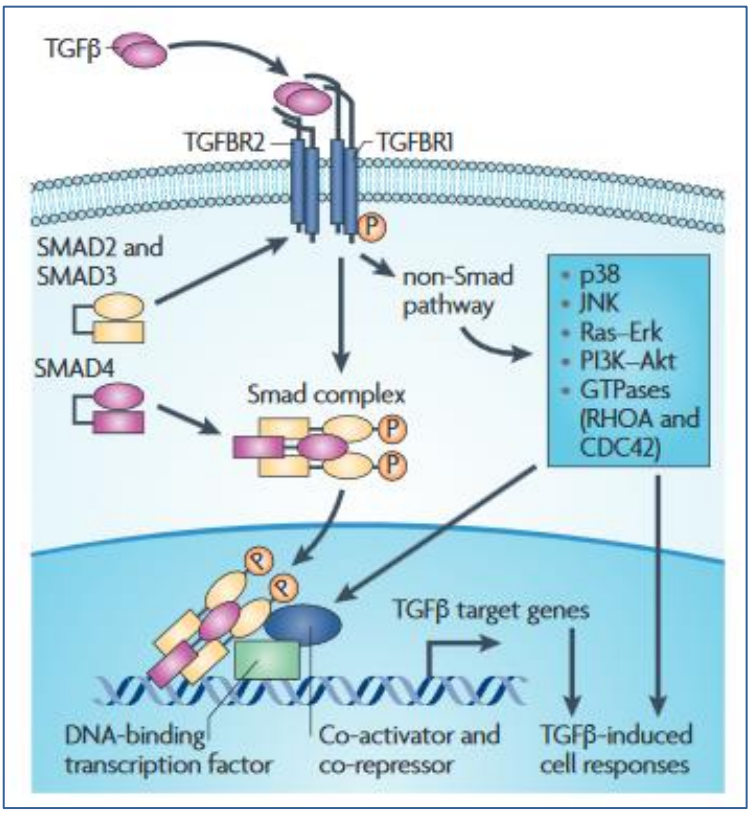

Figure 6. TGF-β canonical and non-canonical signaling pathways. TGF-β binding to a tetrameric complex made of a dimer of TGF-β receptor I and a dimer of TGF receptor II leads to the phosphorylation of TGF-β receptor I and the consequent recruitment of SMAD2 and SMAD3. SMAD2, 3 are in turn phosphorylated and activated. Phosphorylated SMAD2 and SMAD3 form complexes with SMAD4 and translocate into the nucleus where they bind to the DNA and regulate gene expression. In addition to the SMAD pathway, TGF-β may activate other signaling pathways, known as non-SMAD pathways including p38, Jun amino-terminal kinase (JNK), extracellular-signal regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)-AKT, and the RHO GTPase and CDC42 signaling pathways (adapted from Ikushima and Miyazono 2010).

Modulation of TGF-β superfamily signaling occurs on different levels. First, the canonical/SMAD-dependent and the non canonical/SMAD-independent signaling pathways cross-talk at different levels (Zhang, 2017). Further, TGF-β superfamily signaling can be modulated on the receptor level by several mechanisms based on

the interaction of the receptors with the coreceptors betaglycan/TGFβRIII, endoglin, cripto, CD109 and neuropilin, in concert with other cell surface molecules and receptors (Heldin and Moustakas, 2016). Finally, TGF-β receptors activity can be modulated by tuning their endocytosis (Heldin and Moustakas, 2016).

The transcriptional response triggered by TGF-β superfamily ligands is strongly context-dependent (Figure 7). The first group of factors influencing the final cell response is represented by the relative abundance of molecules initiating and controlling the process of signal transduction, i.e., the levels of different TGF-β superfamily ligands, receptors, co-receptors and ligand-traps (Massague, 2012). Further downstream, the abundance of the inhibitory SMAD proteins and the type and intensity of cross-talk between the SMAD and non-SMAD signaling pathways affect SMAD activity (Massague, 2012). A second factor influencing the final cell response is represented by the expression, in a given cell, of the panel of transcription/co–repressor/co-activator factors forming complexes with the SMAD proteins determining which genes are targeted and in which direction, i.e. activating or inhibitory. Finally, the cell epigenetic status, regulating gene accessibility, influences the final response and determines which genes are available for the binding of SMAD complexes and various transcription factors/regulators (Massague, 2012).

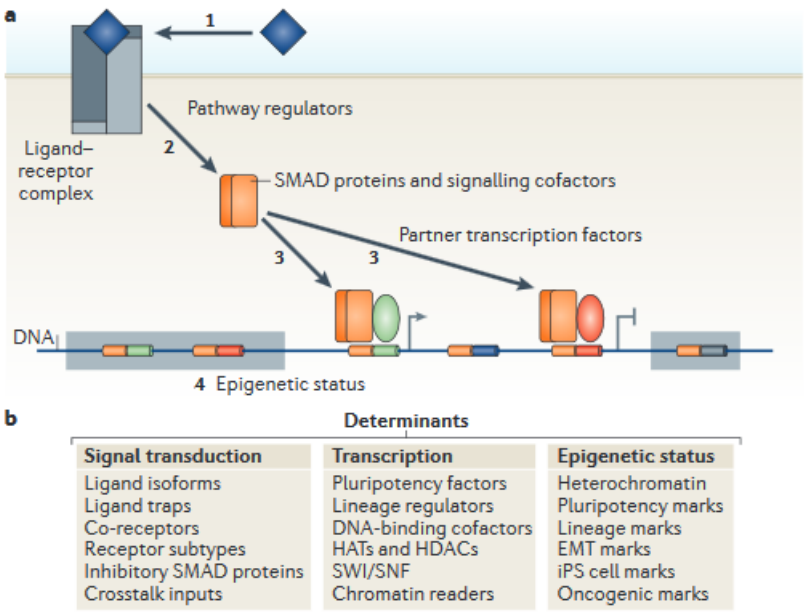

Figure 7. Contextual factors affecting TGF-β signaling and influencing the final transcriptional response. a. A first group of factors affecting the final response of a given cell to TGF-β includes ligands, receptors, co-receptors and ligand-traps (1). More downstream, inhibitory SMAD and the

type and intensity of the cross-talk between the SMAD and non-SMAD signaling pathways affect SMAD activity (2). On a transcriptional level SMAD form complexes with other transcription/co–repressor/co-activator factors (3). Finally, the epigenetic status of the cells determines which genes are available for transcription (4). **b.** The three groups of context determinants affecting TGF-β signaling. EMT, epithelial–to-mesenchymal transition; HATs, histone acetyl transferases; HDAC, histone deacetylase; iPS cell, induced pluripotent stem cell; SWI/SNF, Switch/sucrose non fermentable (adapted from Massague 2012).

3.3.4. TGF-β signaling in glioblastoma

TGF-β signaling plays a central role in the malignant phenotype of glioblastoma by controlling several hallmarks of the disease promoting tumor cell proliferation, migration and invasion, exerting strong immunosuppressive activities, inducing angiogenesis, contributing to tumor chemo- and radio-therapy resistance, and by sustaining glioma stem cell stemness (Figure 8) (Joseph *et al.*, 2013). Several studies demonstrated that glioma cells produce high levels of TGF-β (Wrann *et al.*, 1987; Constam *et al.*, 1992; Sasaki *et al.*, 1995). Moreover, glioma patients show elevated TGF-β2 serum levels, with TGF-β2 levels negatively correlating with patient outcome (Schneider *et al.*, 2006; Bruna *et al.*, 2007). However, recent data from our laboratory challenge this view (Frei *et al.*, 2015). In addition to tumor cells, also the microenvironment, including microglia and tumor vessels, contributes to the release of TGF-β in gliomas (Constam *et al.*, 1992; Wesolowska *et al.*, 2008; Dieterich *et al.*, 2012).

Gliomas are highly invasive, and TGF-β promotes cell invasiveness by inducing the expression of matrix metalloproteinases (MMP) and by suppressing MMP inhibitor (TIMP) expression (Nakano *et al.*, 1995; Wick *et al.*, 2001). Further, TGF-β promotes angiogenesis by regulating the expression of pro-angiogenic factors such as VEGFA (Sanchez-Elsner *et al.*, 2001) and basic fibroblast growth factor (bFGF) (Pepper *et al.*, 1990). Glioblastoma shows high vascular density with abnormal vessels (Wen and Kesari, 2008) and TGF-β2 has been suggested, together with VEGFA, as one of the main mediators to induce abnormal vasculature in glioblastoma (Dieterich *et al.*, 2012). The role played by TGF-β in glioma immune escape has been extensively documented. Interestingly, the TGF-β2 isoform was discovered as the soluble factor released by glioma cells responsible for their immunosuppressive properties and in particular for the inhibition of T helper and T cytotoxic cells functions (Wrann *et al.*, 1987). The immunosuppressive effect of

TGF-β in glioma depends on its action on both the tumor and the immune cells. On the one hand TGF-β2 favors tumor cells immune escape by promoting the downregulation of human leukocyte antigen DR (HLA-DR) (Zuber *et al.*, 1988) and natural killer group 2D (NKG2D) ligand expression (Eisele *et al.*, 2006) in glioma cells. On the other hand TGF-β inhibits the proliferation and cytotoxic activity of T cells (Friese *et al.*, 2004), induces regulatory T cells differentiation and inhibits microglia (Suzumura *et al.*, 1993) and natural killer (NK) cells (Joseph *et al.*, 2013). Indeed, the use of the ALK-5 inhibitor SD-208 in tumor-bearing mice led to an increase in the number of NK, T-cytotoxic lymphocytes and macrophages infiltrating the tumor (Uhl *et al.*, 2004). The inhibition of TGF-β by the neutralizing monoclonal antibody 1D11 in glioma-bearing mice increased the response to tumor vaccination based on the use of glioma-associated peptides, by increasing the response and tumor-recruitment of glioma-associated antigens reactive T-cytotoxic lymphocytes and by reducing the number of Treg infiltrating the tumor (Ueda *et al.*, 2009).

TGF-β signaling has been associated with therapy resistance in gliomas and in particular with radiotherapy resistance. The irradiation of glioma cells led to increased TGF-β secretion and to enhanced invasiveness in one cell line (Canazza *et al.*, 2011), suggesting that TGF-β may play a role in the tumor cell invasiveness observed at the site of irradiation in glioblastoma patients (Han *et al.*, 2015). Treatment of glioma cells with the TGF-β receptor type I/II dual inhibitor LY2109761 increased the cytotoxic effect of radiation treatment with enhanced DNA damage and apoptosis (Zhang *et al.*, 2011).

Different reports have revealed an important role for TGF-β in the maintenance of the stem cell-like properties and the tumorigenic activity of GIC (Ikushima *et al.*, 2009; Gargiulo *et al.*, 2013) with GIC expressing high levels of TGF-β2 (Qiu *et al.*, 2011). In GIC TGF-β promotes the expression of the leukemia inhibitory factor (LIF) increasing GIC self-renewal and reducing cell differentiation (Penuelas *et al.*, 2009). Also, TGF-β, by directly controlling SOX4 expression, promotes SOX2 expression, a known regulator of cell stemness (Ikushima *et al.*, 2009). Notably, it has been reported that TGF-β inhibitors, when used *in vivo*, may target the GIC compartment of the tumor (Anido *et al.*, 2010).

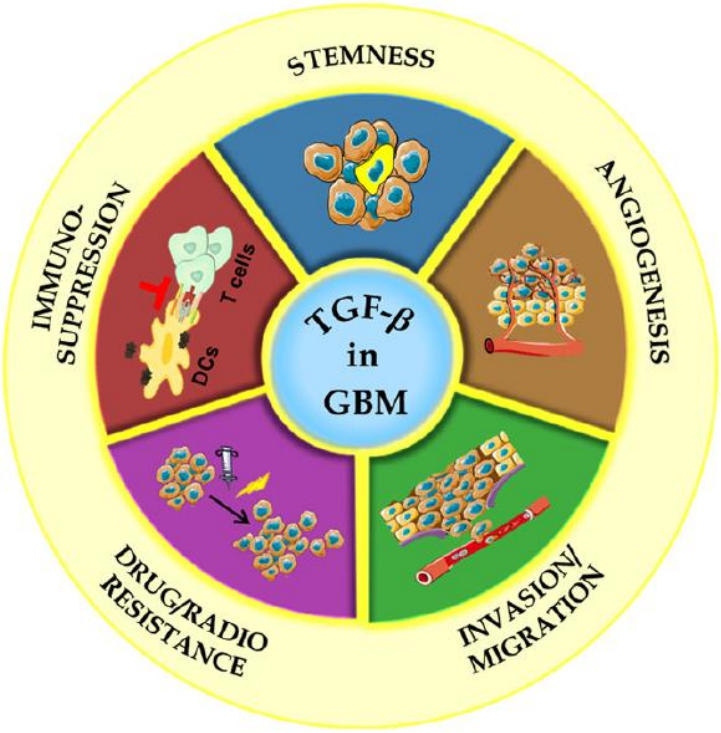

Figure 8. TGF-β control of glioblastoma hallmarks. TGF-β controls the malignant phenotype of glioblastoma by promoting migration and invasion, by exerting strong immunosuppressive functions, by inducing angiogenesis, by sustaining stem cell stemness and by increasing tumor resistance to irradiation and chemotherapy (adapted from Joseph *et al.* 2013).

3.4. PROPROTEIN CONVERTASES

3.4.1 The family of proprotein convertases

Proprotein-convertases subtilisin/kexin (PCSK) form a family of calcium-dependent serine endoproteases related to bacterial subtiliases and yeast kexins. The PCSK family consists of seven core members, PCSK1, PCSK2, PCSK3 known as furin, PCSK4, PSCK5, PCSK6 also known as PACE4 and PCSK7 and two structurally unrelated members, PCSK9 and MTBPS1 (Figure 9) (Seidah and Prat, 2012). The seven PCSK core members are synthesized as pre-pro-proteins (Artenstein and Opal, 2011). The amino-terminal pro-domain regulates folding, activation, transport, and activity of PCSK (Anderson *et al.*, 2002). The pro-domain is followed by the catalytic domain and by the P domain (Figure 9). The last one is conserved among the seven enzymes and regulates the enzymatic activity and its dependency on pH and calcium concentration. Depending on the specific PCSK, the carboxy-terminal region may contain transmembrane and cytosolic domains responsible for the controls of PCSK trafficking through the different subcellular compartments (Artenstein and Opal, 2011). The seven core members process their substrates at single or paired basic residues, (R/K)X_n(R/K) (Duckert *et al.*, 2004). PCSK are involved in the processing of a wide range of substrates, including growth factors, growth factors receptors, hormones, adhesion molecules as well as molecules of viral, bacterial and parasitic origin (Artenstein and Opal, 2011). Protein processing by PCSK has mainly an activating effect, but it can also lead to protein inactivation. PCSK acts in different subcellular compartments, including the trans-Golgi network, cell surface and endosomes as well as the extracellular space (Seidah and Prat, 2012).

About cleaved substrates, some PCSK and mainly PCSK5, PCSK7, furin and PACE4 show redundancy *in vitro*. The selectivity *in vivo* is dependent on the spatial-temporal co-localization of a specific substrate and a determinate PCSK. This co-localization may be passive, like the concomitant presence of furin and TGF-β in the trans-Golgi network or can be active like the co-localization of furin and nodal promoted by the co-receptor crypto (Blanchet *et al.*, 2008)

The main properties of the seven PCSK members, which process their substrate at the consensus recognition sequence which is also present in TGF-β precursor are described in the following.

- PCSK1 and PCSK2 are often co-expressed and usually act in a complementary way (Seidah and Prat, 2012). They are expressed in neural and endocrine cells where they localize in the secretory granules of the constitutive secretory pathway. The substrates processed by PCSK1 and PCSK2 include pro-hormones like pro-insulin, thyrotropin-releasing hormone (TRH) and melanin-concentrating hormone (MCH) and neuropeptide precursors. PCSK1 knockout (Zhu *et al.*, 2002) and PCSK2 knockout mice (Furuta *et al.*, 1997) show severe metabolic and growth problems. PCSK1 knockout mice also show partial perinatal lethality, whereas PCSK2 knockout mice are viable. Human beings with PCSK1 loss are affected by diabetes, hypothyroidism, and hypogonadism (Creemers *et al.*, 2012).

- PCSK4 has been detected only in tissues of the reproductive system and in particular in the ovary, placenta, and testicular germ cells. It is involved in the cleavage of metalloproteases of the a disintegrin and metalloprotease (ADAM) family, pituitary-adenylate cyclase-activating polypeptide and insulin growth factor (IGF)2 (Seidah and Prat, 2012). PCSK4 knockout mice show impaired fertility (Mbikay *et al.*, 1997).

- Two different splice isoforms of PCSK5 have been described, PCSK5A and PCSK5B, which act at the cell surface or ECM. PCSK5A is secreted, whereas PCSK5B is a transmembrane protein which is shed and released extracellularly (Seidah and Prat, 2012). The expression pattern of PCSK5 is quite broad and includes the intestine, the kidney, the ovary and the adrenal cortex. The expression pattern of PCSK5 partially overlaps with the one of PACE4 with which it also shows *in vitro* and *ex vivo* enzymatic redundancy (Seidah and Prat, 2012). PCSK5 specifically processes GDF11 and mice with PCSK5 loss die at birth showing malformations similar to GDF11 knockout mice (Essalmani *et al.*, 2008). In addition to GDF11, PCSK5 is also known to process adhesion molecules like neuronal L1CAM and integrin alpha4 (Seidah and Prat, 2012).

- PACE4 is mainly secreted and acts at the cell surface or in the extracellular matrix. It shows expression overlaps with PCSK5 and *in vitro* and *ex vivo* redundancy with furin and PCSK5 (Seidah and Prat, 2012). The substrates processed by PACE4 include the metalloproteinase ADAM-TS4, the growth factors nodal and lefty and

also some viral glycoproteins (Seidah and Prat, 2012). Mice with PACE4 knockout die as embryos in the 25% of cases depending on the genetic background with cardiac malformations and defects in the right-left patterning (Constam and Robertson, 2000).

- PCSK7 acts only intracellularly being expressed in the trans-Golgi network and in endosomes (Rousselet *et al.*, 2011). PCSK7 show an ubiquitous expression pattern with some partial redundancy with furin, PCSK5 and PACE4 (Seidah and Prat, 2012). PCSK7 processes transferrin receptor-1 being consequently involved in iron metabolism (Guillemot *et al.*, 2013). Besides, it can process pro-BDNF (Wetsel *et al.*, 2013) and some viral proteins and it is involved in the regulation of dopaminergic circuits in the central nervous system. In accordance, mice with a PCSK7 knockout, which are vital, show learning and memory deficits (Wetsel *et al.*, 2013).

- Furin localizes in the trans-Golgi network, at the cell surface, in recycling endosomes and, upon shedding, it can be released into the extracellular environment (Denault *et al.*, 2002). Furin shows ubiquitous expression and substrate redundancy with PCSK5, PCSK7, and PACE4 (Thomas, 2002). Furin processes a high number of substrates including coagulation factors like factor IX and von Willebrand factor, growth factors including members of TGF-β superfamily such as TGF-β (Dubois *et al.*, 2001), BMP4 (Cui *et al.*, 1998) and nodal, IGF1 and its receptor (Khatib *et al.*, 2001), beta-NGF (Molloy *et al.*, 1999), VEGFC and D (Siegfried *et al.*, 2003), PDGF (Siegfried *et al.*, 2005), MMP, members of the tumor necrosis factor (TNF) family and integrins (Thomas, 2002). In addition to endogenous molecules, furin is involved in the processing and activation of pathogen associated molecules. Furin cleavage is required for the activation of some toxins, such as Shiga and Diphtheria toxins (Artenstein and Opal, 2011). Several viruses require furin processing of their envelope glycoproteins to properly fuse with the membrane of host cells, and these include human immunodeficiency virus type I, influenza A viruses, avian influenza viruses like the H5N1 strains, paramoxyvirus, cytomegalovirus and Ebola virus (Artenstein and Opal, 2011).
Furin knockout mice die at the embryonic stage with several severe malformations (Roebroek *et al.*, 1998).

3.4.2 PCSK in disease and their therapeutic targeting

PCSK play fundamental roles in embryogenesis and development as well as in keeping tissue homesteosasis in the adult. The deregulation of their activity associate with different pathological states (Artenstein and Opal, 2011). This prompted the development of PCSK inhibitors with therapeutic purposes (Artenstein and Opal, 2011; Seidah and Prat, 2012).

The lethal phenotype of knockout mice for furin, PACE4 and PCSK5, their ubiquitous expression as well as the redundancy in substrates may require cell- or tissue-specific targeting approaches (Artenstein and Opal, 2011; Seidah and Prat, 2012).

As mentioned above, alterations in PCSK1 and PCSK2 have been associated with endocrinopathies. A molecule blocking glucan processing by PCSK2 and promoting pro-insulin processing by PCSK1 for the potential treatment of diabetes has been suggested (Vivoli *et al.*, 2012). The involvement of PCSK and mainly furin in viral infections led to the evaluation of the antiviral effect of PCSK inhibitors. For some of them, the efficacy *in vitro* as well as *in vivo* in animal models has been proven (Artenstein and Opal, 2011; Seidah and Prat, 2012). PCSK are often deregulated in cancer and metastasis (Artenstein and Opal, 2011; Seidah and Prat, 2012). Furin, PCSK5, PACE4 and PCSK7 are the most commonly up-regulated PSCK in cancers (Cheng *et al.*, 1997; Bassi *et al.*, 2000; Bassi *et al.*, 2001; Mahloogi *et al.*, 2002; Bassi *et al.*, 2003; Bassi *et al.*, 2010; D'Anjou *et al.*, 2011). Increased levels of PCSK1 and PCSK2 have been reported in neuroendocrine tumors (Kajiwara *et al.*, 1999) and lung cancer, too (Creemers *et al.*, 1992). In gliomas PACE4 has been identified as a glioma invasion-associated enzyme (Delic *et al.*, 2012). Also, furin inhibition in astrocytoma cells suppressed their growth and invasiveness (Mercapide *et al.*, 2002) and the use of a PCSK inhibitor revealed that pro-TGF-β is processed by cytoplasmic and secreted PCSK in gliomas (Leitlein *et al.*, 2001). Several approaches have been suggested to inhibit furin in cancer, including small inhibitors and different studies revealed their efficacy in reducing cell growth and invasiveness (Mercapide *et al.*, 2002; Scamuffa *et al.*, 2008). None of these furin inhibitors entered clinical trials (Seidah and Prat, 2012). A phase I clinical trial based on DNA/autologous tumor cell vaccination and involving furin gene silencing brought promising results. In this study, patients with advanced cancers including melanoma, colon and ovarian cancers, were intradermally implanted with

autologous tumor cells that were previously transfected with a plasmid encoding for granulocyte-macrophage colony-stimulating factor (GM-CSF) and for two shRNA targeting furin. The decrease in TGF-β levels in tumor cells as a consequence of furin gene silencing, in combination with high expression of GM-CSF, induced an effective tumor response and led to prolonged survival (Senzer *et al.*, 2012).

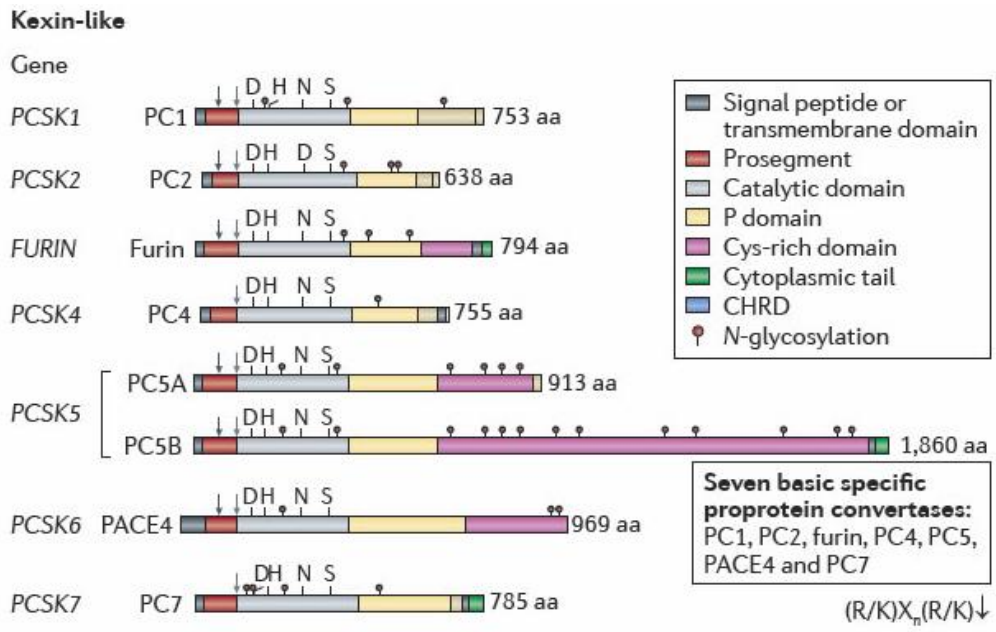

Figure 9. The family of PCKS. The primary schematic structure, domain composition and glycosylated sites of PCSK are indicated. The consensus sequence of the seven PCSK core members is reported in the right lower corner. PCSK, proprotein convertases subtilisin/kexin; PC, proprotein convertases; CHRD, Cys–His-rich domain (adapted from Seidah and Prat 2012).

3.5. FIBRONECTINS (FN)

3.5.1 The FN isoforms containing the extra-domains A and B

FN are high molecular mass adhesive glycoprotein of the extracellular matrix (Pankov and Yamada, 2002). They are present in the ECM of solid tissues as well as in body fluids. FN are modular proteins characterized by the repetition of three different domains, named FN type I, type II and type III domains. FN molecules form dimers by two disulfide bridges in the carboxy-terminal region (Figure 10). They are involved in the regulation of several cell functions including cell growth and differentiation, cell adhesion, cell migration, establishment and maintenance of normal cell morphology and hemostasis (Hynes, 1990). These functions are mediated by the interaction of FN with other components of the ECM, like collagen, heparin and chondroitin sulfate, with cellular integrins and with growth factors including VEGF and TGF-β. FN are encoded by a single gene on chromosome 2 (Zardi *et al.*, 1982), but up to twenty isoforms may be generated by post-translational modifications such as glycosylation as well as by alternative splicing mechanisms (Pankow Yamada 2002). Alternative splicing can affect three different domains: the extra domain A (EDA), the extra-domain B (EDB) and the type III connecting sequence (IIICS) (Schwarzbauer, 1991). Alternative splicing of FN is regulated by several factors including pH and cytokines and TGF-β has been identified as one of the most important regulators of this process, leading to an increase in EDA+FN and EDB+FN levels in several cell types (Balza *et al.*, 1988; Borsi *et al.*, 1990; Viedt *et al.*, 1995). The inclusion of these domains in FN molecules leads to the introduction of additional integrin binding sites, but it also leads to conformational modifications with exposure of otherwise cryptic sequences finally affecting ECM matrix assembly and cell signaling (Carnemolla *et al.*, 1992; Leahy *et al.*, 1996; Bencharit *et al.*, 2007).

The FN isoforms containing the EDA and EDB domains are abundantly expressed in fetal tissues, and during development, by contrast, their expression is restricted in the adult (White *et al.*, 2008). EDA+FN in the adult is expressed in neo-forming vessels, in inflamed and fibrotic tissues, in wounds and tumors. The EDA domain directly interacts with the integrins α9β1 (Shinde *et al.*, 2008) and α4β1 (Shinde *et al.*, 2015) and with toll-like receptor 4 (TLR4). The presence of EDA domain in FN molecules also increases binding to the integrin α5β1 (Manabe *et al.*, 1997). By

interacting with the integrins mentioned above, EDA+FN modulates adhesion of the cell to the ECM (Shinde *et al.*, 2008; Shinde *et al.*, 2015). By controlling the activation of TGF-β and by promoting α-smooth muscle cell actin (α-SMA) expression EDA+FN mediates activation and differentiation of fibroblasts into myofibroblasts and thus plays an important role in wound healing (Muro *et al.*, 2003) and fibrogenesis (Serini *et al.*, 1998; Muro *et al.*, 2008; White *et al.*, 2008; Kohan *et al.*, 2010; Kawelke *et al.*, 2011). The interaction of EDA+FN with TLR4 has been associated with immune and pro-inflammatory functions, such as the enhancement of cytotoxic responses (Julier *et al.*, 2016) and the activation of macrophages (Doddapattar *et al.*, 2015). In addition, EDA+FN is involved in morphogenesis of lymphatic vessels (Bazigou *et al.*, 2009) and in sustaining the stemness properties of embryonic stem cells (Losino *et al.*, 2013), of colorectal cancer stem cells (Ou *et al.*, 2013) and in promoting colorectal cancer metastasis. Importantly, this may be due to its role in inducing epithelial-to-mesenchymal transition (Ou *et al.*, 2014). The high expression of EDA+FN in chronically inflamed tissues has prompted the development of recombinant antibodies specific for EDA fused with the cytokines IL-4 and IL-10. These molecules, allowing targeting (via EDA) and treatment (via IL-4 or IL-10) of *in vivo* chronic inflamed tissues, are currently tested in clinical trials in patients with rheumatoid arthritis and endometriosis (Galeazzi *et al.*, 2014).

The FN isoform containing the EDB domain is expressed in the adult in tissues undergoing remodeling and angiogenesis, being abundantly expressed in neoplastic tissues (Zardi *et al.*, 1987; Kaczmarek *et al.*, 1994; Kosmehl *et al.*, 1996). During angiogenesis, EDB+FN accumulates around newly formed vessels, and it is thus considered a marker of this process (Castellani *et al.*, 1994). Glioblastoma vessels show abundant expression of EDB+FN. Also, it has been shown that in gliomas the percentage of vessels positive for EDB+FN correlates with tumor grade (Castellani *et al.*, 2002). The biological functions of EDB as well as its potential binding partners are not yet clear. Recently, a role for EDB in the regulation of VEGF expression in endothelial cells has been suggested (Khan *et al.*, 2005; Chen *et al.*, 2012) as well as a role in promoting phagocytosis in an αvβ3-dependent manner (Kraft *et al.*, 2016). The high expression of EDB+FN in tumors and tumor vasculature has prompted the development of EDB+FN-specific antibodies for diagnostic and therapeutic purposes. In particular the EDB-specific recombinant antibody L19 (Pini *et al.*, 1998) is currently under investigation in clinical trials in cancer patients as

fusion protein with the cytokines IL-2 (Carnemolla *et al.*, 2002; Zegers *et al.*, 2015) and TNF-α (Borsi *et al.*, 2003; Danielli *et al.*, 2015) as well as radio-labeled molecule (Poli *et al.*, 2013).

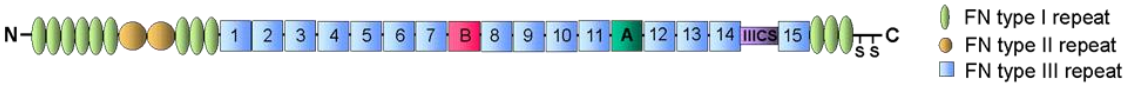

Figure 10. The modular structure of fibronectin. Fibronectin (FN) is a modular protein characterized by the repetition of three different types of domains, known as FN type I, II and III repeats. Alternative splicing can affect three different regions: the extra-domain A (indicated in green), the extra-domain B (ED-B) (indicated in red) and the type III connecting sequence (IIICS) (indicated in purple). FN form covalent homo- or hetero-dimers owed to two disulfide bonds in the carboxy-terminal region.

3.5.2 FN in vascular morphogenesis and plasticity

Fibronectins evolutionary appeared with the appearance of an endothelium–lined vasculature (Hynes and Zhao, 2000). Genetic studies performed in mice, zebrafish and xenopus attested a role for fibronectin in vascular morphogenesis and cardiovascular development (Astrof and Hynes, 2009). Indeed FN-null embryos die because of lethal vascular defects at embryonic (e) day 8-8.5 (George *et al.*, 1993; George *et al.*, 1997; Francis *et al.*, 2002). Several functions of FN in vascular morphogenesis are dependent on the interaction of FN with integrins and mainly with the integrin α5β1. FN-α5β1 binding is mediated by the RGD sequence located on the FN type III domain 10 and by a synergy site belonging to the FN type III domain 9 (Leahy *et al.*, 1996). Embryos of knockout mice for integrin α5 and of transgenic mice in which the RGD sequence was substituted by a RGE sequence died at days e9.5-10 and e10, respectively, and showed severe vascular defects, even if slightly milder, to the ones reported for FN knockout mice (Yang and Hynes, 1996; Takahashi *et al.*, 2007). These studies attest a central role for FN and α5β1 in vascular morphogenesis. The higher severity of the phenotype of FN knockout mice suggests that other FN functions independent of the interaction with α5β1 are involved. The specific role of the EDA and EDB splice variants of FN in vascular morphogenesis is less clear. EDA/EDB double knockout mice embryos die between days e9.5 and e10.5 from severe cardiovascular defects (Astrof *et al.*, 2007). By

contrast, single EDA and EDB knockout mice are viable and fertile (Fukuda *et al.*, 2002; Muro *et al.*, 2003). EDA/EDB double null mice embryos show sheets of endothelial cells instead of vascular networks (Astrof *et al.*, 2007). Interestingly, the phenotype of these double null mice is similar to the ones reported for mice carrying deletions in different molecules involved in vascular morphogenesis and plasticity, i.e. VEGF-A, angiopoietin (Ang)1, bFGF and TGF-β1 (Dickson *et al.*, 1995; Ferrara *et al.*, 1996; Lee *et al.*, 2000). Several hypotheses exist for the potential mechanisms responsible for the phenotype of EDA/EDB double knockout mice and its similarity with knockout mice of the above-mentioned growth factors including a potential role of the EDA and EDB domains in regulating growth factor signaling (Astrof and Hynes, 2009). FN-α5β1 interactions are involved in the signaling of various growth factors including PDGF-BB, EGF, bFGF and TGF-β1 (Astrof and Hynes, 2009). Beyond, FN mediates TGF-β1 signaling through SMAD1,5,8 by promoting the formation of an ALK-1/endoglin complex in human microvascular endothelial cells (Tian *et al.*, 2012). A potential role for the EDA and EDB domains in modulating TGF-β signaling has been hypothesized (Astrof and Hynes, 2009).

Endothelial cells show high phenotypic plasticity which is a consequence of their exposure to a high variety of stimuli, represented by both soluble factors, such as cytokines and growth factors and mechanical forces (Dejana *et al.*, 2017). The hallmark of endothelial cell plasticity is endothelial-to-mesenchymal transition (EndMT). The process of EndMT is characterized by the progressive loss of endothelial properties and gain in mesenchymal properties (Goumans *et al.*, 2008). Endothelial cells undergoing EndMT show cytoskeleton rearrangements, loss of cell polarity and alteration in endothelial junctions, paralleled by the acquisition of a fibroblast morphology, increased FN and collagen secretion and an increase in motility, proliferative, migratory, invasive and thrombogenic abilities (Kalluri and Weinberg, 2009). Endothelial cells undergoing EndMT acquire properties of different mesenchymal-lineage cell types such as fibroblasts, smooth muscle cells, chondrocytes or adipocytes (Dejana *et al.*, 2017). During EndMT endothelial cells start to express mesenchymal markers such as α-SMA and neural (N)-cadherin and progressively loose the expression of endothelial markers such as CD31 and vascular endothelial (VE)-cadherin (van Meeteren and ten Dijke, 2012).

EndMT occurs during development, where it plays a fundamental role in cardiac development (Thiery and Sleeman, 2006) generating cardiac fibroblasts and smooth muscle cells, as well as in postnatal settings. Postnatal EndMT is associated with different pathological conditions including tissue fibrosis (Piera-Velazquez *et al.*, 2011), atherosclerosis (Chen *et al.*, 2015) and cancer (Zeisberg *et al.*, 2007; Xiao *et al.*, 2015). In cancer, EndMT contributes to the generation of cancer-associated fibroblasts and thus favors tumor progression and invasion. Also, by reducing endothelial cell junctions, it facilitates tumor cells intravasation and metastatic dissemination (Dejana *et al.*, 2017).

Several signaling pathways promote EndMT with TGF-β signaling playing a central role in the regulation of plasticity of endothelial cells. In endothelial cells, TGF-β can activate both the ALK-5/SMAD2,3 and ALK-1/SMAD1,5,8 signaling pathways, with the ALK-5/SMAD2,3 pathway promoting endothelial cells quiescence and the ALK–1/SMAD1,5,8 pathway stimulating proliferation and migration of endothelial cells. It has been proposed that the balance between these two signaling pathways as well as their cross-talk affects the activation state of endothelial cells (Goumans *et al.*, 2002). Different studies show that TGF-β may induce the process of EndMT in endothelial cells, with a preferential involvement of SMAD3-dependent signaling (van Meeteren and ten Dijke, 2012). TGF-β induces the expression of transcription factors such as Snail, Slug and Twist that in turn regulate the expression of several genes involved in the process of EndMT (Medici *et al.*, 2011). Also, TGF-β can directly induce the expression of proteins which are considered as markers of EndMT (Dejana *et al.*, 2017), including FN. Recently, it has been shown that TGF-β induces EndMT in endothelial cells derived from freshly dissociated human glioblastoma in an ALK-5-dependent manner (Krishnan *et al.*, 2015).

Glioblastomas are characterized by a high vascular density, vascular abnormalization and the formation of glomeruloid vascular structures (Wen and Kesari, 2008). Among the genes up-regulated in glioblastoma tumor vasculature are several EndMT markers including fibronectin. Beyond, SMAD3/SMAD4 complexes are preferentially localized in the vasculature or the perivascular areas, suggesting a direct contribution of TGF-β signaling in the expression of genes responsible for glioblastoma vasculature abnormalization (Dieterich *et al.*, 2012). Recently, it has been demonstrated that glioblastoma-derived endothelial cells, undergoing EndMT, acquire a fibroblast-like phenotype, express high amount of ECM proteins including

FN, become more invasive and contribute to glioblastoma vessels abnormalization (Huang *et al.*, 2016).

4. MATERIAL AND METHODS

Cell culture and reagents

LN-229 and LN-308 kindly provided by N. de Tribolet (Lausanne, Switzerland) were cultured in DMEM (Gibco/Thermo Fisher, Madison, WI) supplemented with 1% L-glutamine (Gibco) and 10% FBS (Gibco). The human GIC T-325, T-269, ZH-161, S-24 and ZH-305 (Lemke *et al.*, 2014) were cultured in phenol red-free neurobasal medium (NBM) (Gibco) supplemented with 2% B-27 without vitamin A (Gibco) (20 µl/ml), 1% L-glutamine (Gibco) and fibroblast growth factor and epidermal growth factor (FGF/EGF) (20 ng/ml each) (PeproTech, London, UK). Treatment of cells was performed in NBM containing 1% L-glutamine and FGF/EGF.

Lentivirally-mediated gene silencing of furin was performed as described (Szabo *et al.*, 2016) using a furin specific shRNA (V3LHS_310002) cloned in a pGIPZ lentiviral vector and the respective pGIPZ vector carrying a non-targeting control (RHS4349) (Dharmacon, Lafayette, CO).

Transient gene silencing was performed by electroporation (Neon Transfection System, Invitrogen/ Thermo Fisher) using 100 nM of the following ON-TARGET plus, siRNA SMART pools from Dharmacon: TGF-β1 (L-012562-00), SMAD2 (L-003561-00), SMAD3 (L-020067-00), SMAD4 (L-003902-00), ERK1 (L-003592-00), ERK2 (L-003555-00) and non-targeting control (D-001810-10).

Human cerebral microvascular endothelial cells (hCMEC/D3) were kindly provided by P.C. Couraud (Paris, France). The human glioblastoma-derived endothelial cells ZHE-464, ZH-483-2 and ZHE-459 were previously reported (Krishnan *et al.*, 2015). Endothelial cells were cultured in EBM-2 medium (CC-3156, Lonza, Walkersville, MD), containing 0.1 M HEPES (Gibco), 1% v/v CD lipid concentrate (Gibco) and endothelial growth supplements (EGM-2-CC4176, Lonza).

Transient gene silencing in endothelial cells was performed by using the Lipofectamine RNAiMAX reagent (Invitrogen) and the above described siRNA specific for SMAD2,3,4 and siRNA control. For EDA+FN and EDB+FN the following siRNA provided by Dharmacon and used at the final concentration of 10 nM were used: 1) EDA+FN: pool of siEDA1 5'- GGTTCTGAGTACACAGTCA-3' and siEDA2 5'- GGTTCTGAGTACACAGTCA - 3'; 2) EDB+FN: pool of four siRNA previously described (Khan *et al.*, 2005).

Cells were treated in EBM-2 medium without supplements.

Human peripheral blood mononuclear cells (PBMC) were prepared from healthy donor blood and cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 20 mM L-glutamine, 50 µM β-mercaptoethanol (Sigma, St. Louis, MO), 10 U/ml penicillin (Sigma), 10 µg/ml streptomycin (Sigma), 10 mM sodium-pyruvate (Sigma), and 1x Minimum essential medium nonessential aminoacids (Gibco). Media from GIC seeded at 10^7 in 10 ml NBM for 72 h were concentrated 2.5-fold prior to the addition to the PBMC. On day 0 PBMC were treated with 3 µg/ml concavalin A (Sigma), 20 U/ml IL-2 (PeproTech) and GIC conditioned media. At day 3 treatment with IL-2 and exposure to GIC media were repeated, and at day 6 addition of IL-2 was repeated. On day 7 cells were analyzed by flow cytometry.

The following reagents were used: PCSK inhibitor Decanoyl-RVKR-CMK (3501, Tocris Bioscience, Bristol, UK), recombinant human TGF-β2 (R&D Systems, Minneapolis, MN), SD-208 (Scios, Fremont, CA), mitogen-activated protein/extracellular signal-regulated kinase (MEK)1/2 inhibitor U0126 (9903, Cell Signaling Technology, Danvers, MA), JNK inhibitor SP600125 (Sigma-Aldrich). The human fibronectin recombinant fragments used in this study were previously described (Carnemolla *et al.*, 1996).

Immunoblot analysis

Whole cell lysates or concentrated conditioned media in denaturing and reducing conditions were separated by using 10% or 12% polyacrylamide gels and transferred to nitrocellulose membranes (Amersham/Ge Healthcare Life Science, Arlington Heights, IL). After staining with red ponceau (ApplChem, Darmstadt, Germany) for equal loading, membranes were blocked with 5% skimmed milk or serum bovine albumin (ApplChem) and treated with primary antibodies. Protein bands were visualized with horseradish peroxidase (HRP)-coupled secondary antibodies followed by enhanced chemoluminescence (Pierce/Thermo Fisher, Madison, WI). Primary antibodies used were anti-furin (sc-20801, Santa Cruz Biotechnology, Dallas, TX), anti-β-actin (sc-1616, Santa Cruz), anti-human LAP/TGF-β1 (AF-246-NA, R&D Systems), anti-TGF-β1 (G1221, Promega, Madison, WI), anti-TGF-β2 (ab36495, Abcam, Cambridge, UK), anti-GAPDH (EB07069, Everest Biotech, Ramona, CA) and antibodies from Cell Signaling Technology specific for SMAD1 (9743), phosphorylated SMAD1/5 (Ser463/465)

(9516), SMAD2 (3122), phosphorylated SMAD2 (Ser465/467) (3108), SMAD3 (9513), phosphorylated SMAD3 (Ser423/425) (9520), SMAD4 (9515), p44/42 MAPK (ERK1/2) (9102), phosphorylated p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (9106), AKT (9272), phosphorylated AKT (Ser473) (9271), p38 MAPK (9212), phosphorylated p38 MAPK (Thr180/Tyr182) (9211). FN, EDA+FN and EDB+FN were detected by using the IST4 (Sekiguchi *et al.*, 1985), IST9 (Borsi *et al.*, 1987) and C6 (Balza *et al.*, 2009) antibodies, respectively. Secondary antibodies were HRP-coupled goat anti-rabbit (sc-2004), donkey anti-goat (sc-2033) (Santa Cruz Biotechnology) or sheep anti-mouse antibodies (NA931V, Ge Healthcare UK limited, Amersham, UK).

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed by using the ΔC_{TT-} method with ARF1 as a housekeeping gene. The following primers were used: PCSK1 for 5'-CCTGGAAGCAAACCCAAATC-3', PCSK1 rev 5'-CAGACAACCAGGTGCTGCATA-3', PCSK2 for 5'-AATGCCGAAGCAAGTTACGACT-3', PCSK2 rev 5'-CCCGTGGCTGTTAAACCAGT-3', PCSK4 for 5'-AGCAAAGAGAACCGGCACG-3', PCSK4 rev 5'-TCGATGACATCGGTGATGGT-3', PCSK5 for 5'-GGCCAACAGGCTATTTGATCA-3', PCSK5 rev 5'-GCTCTTTCTCCCCAGCAATG-3', PCSK6 for 5'-CACCTGCTAGTGAAGACATCC-3', PCSK6 rev 5'-AACGAGAGCTTCTGCGTCCAC-3', PCSK7 for 5'-CCACCCGGTATGAGGATCG-3', PCSK7 rev 5'-AAACCGTGCTGGTGGCTATG-3', furin for 5'-GGACTAAACGGGACGTGTACC-3', furin rev 5'-AGGCCGCCTTCACATTCAG-3', FN for 5'-TACACTGGGAACACTTACCG-3', FN rev 5'-CCAATCTTGTAGGACTGACC-3', EDA+FN for 5'-GGAGAGAGTCAGCCTCTGGTTCAG-3', EDA+FN rev 5'-TCTGCAGTGTCTTCTTCACC-3', EDB+FN for 5'-TCAAGGATGACAAGGAAAGTG-3', EDB+FN rev 5'-AATAATGGTGGAAGAGTTTAGC-3', ID1 for 5'-CCAGCACGTCATCGACTACA-3', ID1 rev 5'-GGAACGCATGCCGCC-3', PAI-1 for 5'-CAGAAAGTGAAGATCGAGGTGAAC-3', PAI-1 rev 5'-GGAAGGGTCTGTCCATGATGAA-3', ARF1 for 5'-GACCACGATCCTCTACAAGC-3' and ARF1 rev 5'-TCCCACACAGTGAAGCTG-3'.

Furin-specific activity

Cellular and extracellular furin-specific activity (FSA) was measured as previously described (Bourne and Grainger, 2011) using the anti-furin antibody MAB15032 (R&D Systems) as capturing antibody in lysates and conditioned media of cells seeded at a density of $5x10^6$ cells in 7 ml NBM supplemented with L-glutamine and FGF/EGF for 48h. FSA is expressed as the fluorescence intensity (λexc=380nm, λem=460nm) normalized to protein concentration as determined by Bradford assay (Bio-Rad, Hercules, CA). FSA was measured in concentrated conditioned media mixed with the appropriate amount of reaction buffer and analyzed in the same manner as previously described for the lysates (Bourne and Grainger, 2011).

Flow cytometry

APC-CD25 (17-0259) and PE-FOXP3 (12-4776) antibodies were from eBioscience (San Diego, CA), FITC-CD4 (55346) from BD Biosciences (San Jose, CA). FOXP3 Afixation/permeabilization kit (eBioscience) was used for FOXP3 staining. Cells were analyzed with FACS Verse (BD biosciences) and data analyzed with FlowJo software. Fluorescence Minus One (FMO) controls were performed by using the following fluorochrome-conjugated isotype control antibodies from BD biosciences: FITC-mouse IgG1, κ (555748), APC-mouse IgG1, κ (555751) and PE-rat IgG2A, κ (553930).

Enzyme-linked immunosorbent assay (ELISA)

BRAND Immunograde 96 wells plates (Sigma-Aldrich) were coated with 20 µg/ml gelatin in PBS overnight at room temperature, washed with PBS and blocked with 2% BSA in PBS. Conditioned cell culture media were tested at different dilutions in 2% BSA in PBS. FN, EDA+FN and EDB+FN were detected by using the IST4, IST9 and C6 antibodies, respectively, diluted in 2% BSA in PBS. The minimal cross-reactivity anti-mouse IgG-HRP antibody (BioLegend, San Diego, CA) was used as secondary antibody. 3,3', 5,5' tetramethylbenzidine (TMB) (BD Bioscience) was used as reaction substrate. Data are expressed as the values of absorbance at 450 nm normalized to protein concentration as determined by Bradford Assay (Bio-Rad).

Immunohistochemistry

For immunohistochemical analysis 5 µm cryostat sections of ten human glioblastoma samples were used. Sections were stained using the primary antibodies IST-4, IST-9 and C6, the peroxidase mouse IgG Vectastain ABC kit (PK-4002, Vector Laboratories, Burlingame, CA) and the DAB substrate (Peroxidase blocking, Dako-Agilent, Santa Clara, CA).

Statistical analysis

All experiments were performed at least twice and in triplicates. Statistical analysis was performed by using the GraphPad Prism 5 program. Data are shown as the mean ± the standard deviation (SD). The statistical significance of the data was determined by performing unpaired Student t-test or one-way Anova followed by Tukey's post hoc test 95% CI.

5. AIMS OF THE DOCTORAL THESIS

Project 1.

Given the central role played by TGF-β in the malignant phenotype of glioblastoma and in GIC, the first goal of the project was to define the relevant PCSK for pro-TGF-β processing with a particular focus on GIC. As furin was one of the main PCSK expressed in GIC, the aims of the project were:

- To define the role played by furin in pro-TGF-β processing and TGF-β signaling performing a stable selective gene silencing of furin in GIC;

- To investigate the regulation of furin levels with emphasis on TGF-β-dependent pathways as previous reports showed that TGF-β may control furin expression.

Project 2.

Since TGF-β and EDA+FN and EDB+FN are highly expressed in glioblastoma vasculature, the main goal of the project was to define the potential bi-directional interaction of TGF-β and FN in glioblastoma, focusing on endothelial cells. More specifically the aims of the project were:

- To investigate the potential regulation EDA+FN and EDB+FN expression by TGF-β;

- To investigate the potential regulation of TGF-β signaling by EDA+FN and EDB+FN.

6. RESULTS AND DISCUSSION

6.1 Extracellular signal-regulated kinase 1 (ERK1) mediates the autocrine positive feedback loop of TGF-β and furin in glioma-initiating cells

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Experimental contribution: I performed the experiments under the supervision of Isabel Burghardt and Michael Weller.

6.1.1. Results

Seven of the nine PCSK, i.e. PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin, process their substrate at the consensus recognition sequence $(R/K)X_n(R/K)\downarrow$ which is also the recognition site for processing TGF-β. We first investigated mRNA expression levels of these enzymes in a panel of five human GIC: T-325, T-269, ZH-161, S-24 and ZH-305. Furin was expressed in all cell lines and one of the most abundant PCSK (Figure 11A). Furin was detectable intracellularly in cell lysates as a double band of around 100 kDa, corresponding to the two glycosylated/sialylated forms of mature furin (Denault *et al.*, 2002) except for T-269 where only the higher molecular mass isoform was detected. In the conditioned media furin was detectable as a major band with an apparent molecular mass of around 90 kDa (Figure 11B).

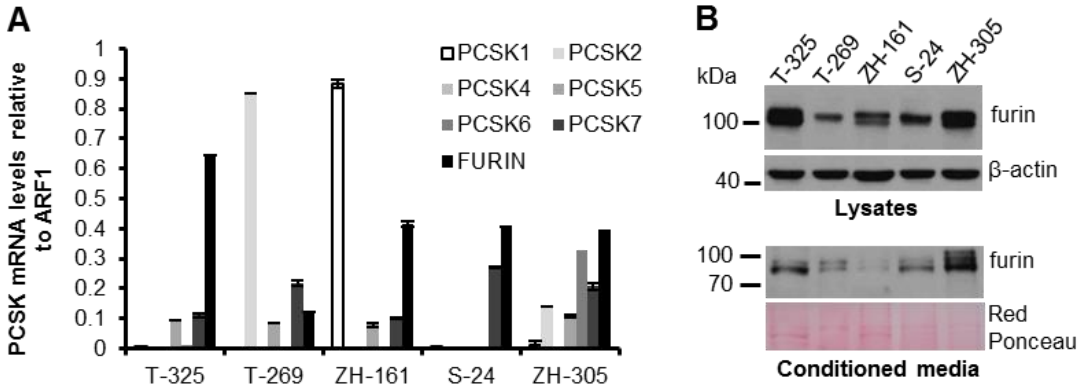

Figure 11. Expression of proprotein convertases subtilisin/kexin (PCSK) in GIC. A. RT-PCR analysis of PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin. **B.** Analysis of furin levels by immunoblot in the lysates and conditioned media.

To investigate the processing of TGF-β1/2 by furin, lentiviral gene silencing of furin was performed in T-325, ZH-161 and ZH-305 cells, further referred to as shfurin cells (Figure 12). Furin gene silencing did not affect any other PCSK with the exception of PCSK6 in ZH-305 shfurin cells (data not shown). Decreased protein levels of both cellular and secreted furin (Figure 12) and a reduction in cellular and extracellular furin-specific activity (FSA) (Figure 13) were confirmed in all furin knockdown cell lines. We then analyzed TGF-β1/2 processing in shfurin cells. For TGF-β1 we used an antibody to the N-terminus of TGF-β1 detecting both pro-TGF-β1 (55kDa) and the latency associated peptide (LAP) of TGF-β1 (37 kDa) and an antibody to the C-terminus detecting mature TGF-β1 (12.5 kDa) (see ZH-

161/siTGF-β1 as a control, Figure 12). For TGF-β2 we used an antibody reacting with the C-terminus of TGF-β2 detecting both pro-TGF-β2 (55 kDa) and mature TGF-β2 (12.5 kDa). Furin gene silencing resulted in increased levels of pro-TGF-β1 and correspondingly decreased levels of the two respective processing products LAP/TGF-β1 and mature TGF-β1 in ZH-161 and ZH-305, with TGF-β1 being not detectable in T-325 (Figure 12). In T-325 and ZH-305, showing detectable TGF-β2 protein levels, furin gene silencing increased levels of pro-TGF-β2 and decreased levels of mature TGF-β2. The effects of furin gene silencing on pro-TGF-β1/2 processing were similar to those obtained with the pan-proprotein convertase inhibitor decanoyl-RVKR-CMK (PCSK inhibitor), suggesting that furin is the main PCSK involved in pro-TGF-β1/2 processing here. Notably, in T-325 and ZH-161 pro-TGF-β processing takes place to a remarkable extent extracellularly as pro-TGF-β levels increased in shfurin cells and upon addition of the PCSK inhibitor in the conditioned media, but remained unchanged in the lysates. In ZH-305, the levels of pro-TGF-β2 increased both in cell lysates and in conditioned media following furin gene silencing, suggesting that pro-TGF-β2 processing takes place in part in the intracellular compartment in ZH-305 cells, too (Figure 12).

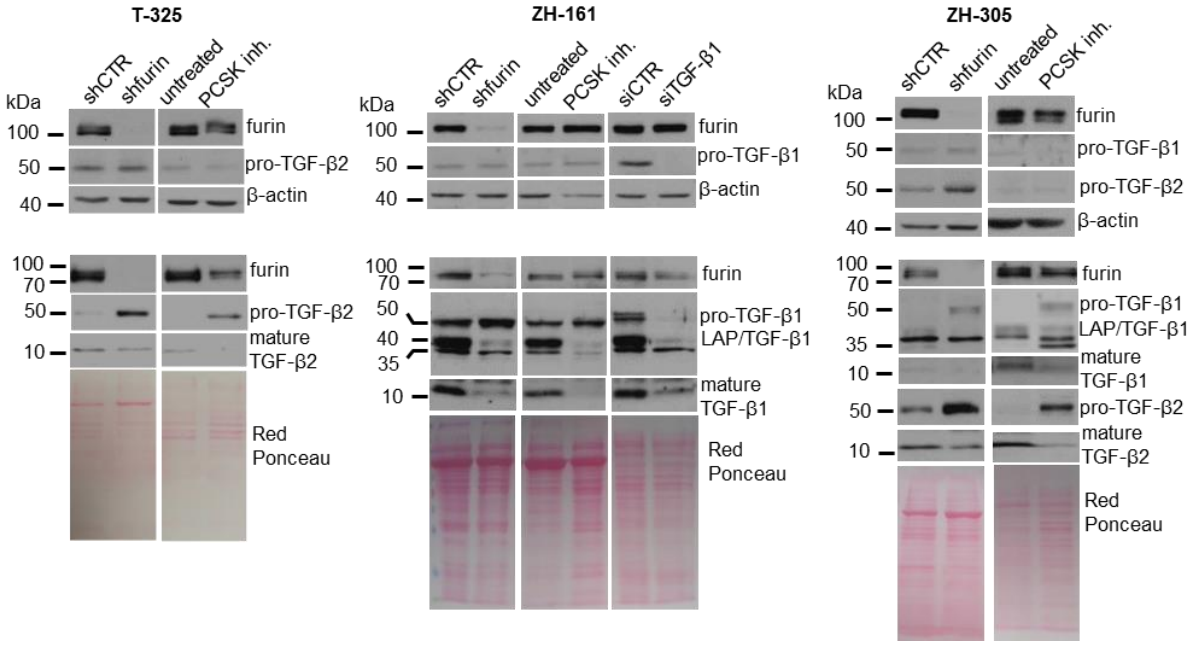

Figure 12. Furin processes TGF-β in GIC. Protein levels of furin and precursor forms of TGF-β1/2 were determined in cell lysates (upper panels) and conditioned media (lower panels) of furin-silenced or control cells or cells treated with 25 μM PCSK inhibitor Decanoyl-RVKR-CMK for 48h. Precursor and mature forms of TGF-β1/2 were determined in the conditioned media of control, furin-depleted and PCSK inhibitor-treated cells.

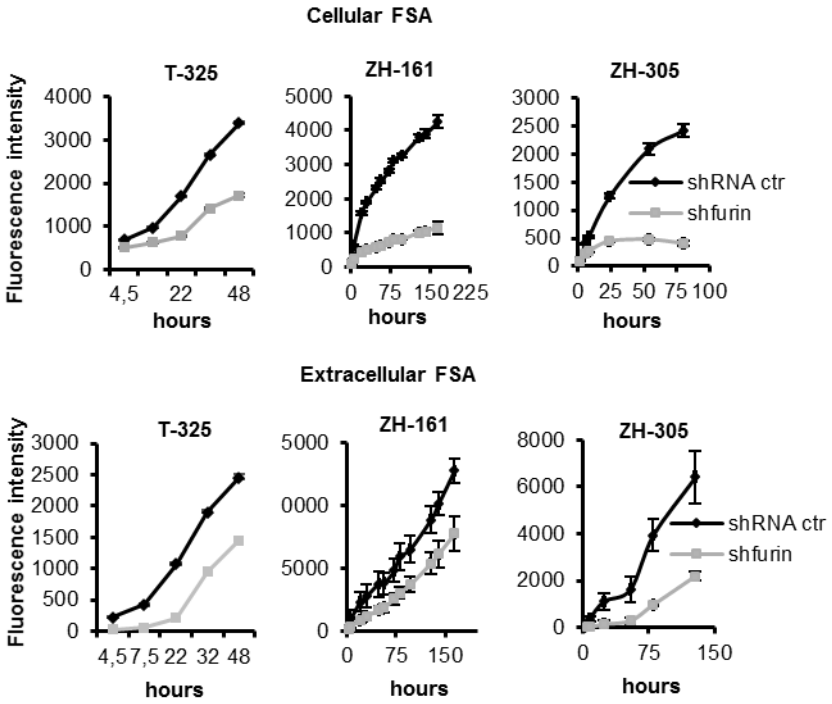

Figure 13. Furin-specific activity (FSA) in GIC with furin gene silencing. Cellular and extracellular FSA was measured in lysates and conditioned media of control or furin-depleted cells.

To confirm that reduced TGF-β levels affect down-stream signaling activities, we analyzed the levels of phosphorylated SMAD (pSMAD)2, pSMAD3 and pSMAD1,5 as readouts for the activation of canonical TGF-β signal transduction, and of phosphorylated AKT (Ser473) (pAKT (Ser473)), ERK1/2 (pERK1/2) and p38 (pp38) reflecting non-canonical TGF-β signaling (Figure 14). All sh-furin cells showed reduced levels of pSMAD2 and pSMAD3. In ZH-161 and ZH-305 pSMAD1,5 was also reduced. The phosphorylation of AKT (Ser467) and p38 was reduced in T-325 shfurin cells, and ZH-305 shfurin cells showed a reduction in the phosphorylation of ERK and p38 as well.

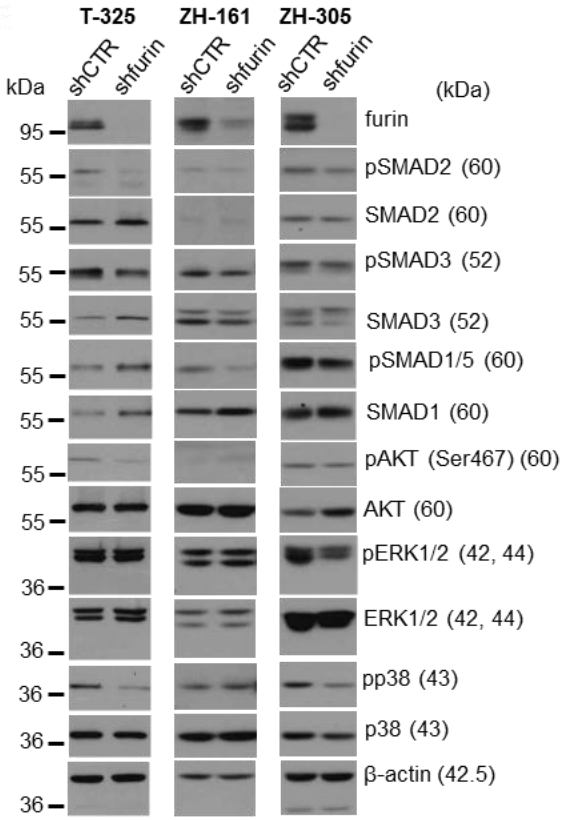

Figure 14. TGF-β signaling in GIC with furin gene silencing. Total SMAD1,2,3, phosphorylated SMAD1,2,3,5, total and phosphorylated AKT, ERK1/2 and p38 levels were determined in control or furin-depleted cell lysates by immunoblot. In brackets the predicted molecular mass is indicated.

TGF-β exerts several immunosuppressive functions, including the promotion of FOXP3+ Treg cells. Previous studies have demonstrated that GIC induce Treg generation *in vitro* (Wei *et al.*, 2010). Accordingly, we treated human PBMC with GIC-conditioned media and confirmed the induction of CD4+CD25+FOXP3+ cells: conditioned media of ZH-161 cells increased the fraction of CD4+CD25+FOXP3+ cells from 8.9 to 14.8 ± 0.1%. TGF-β2 treatment was used as a positive control. To test whether the inhibition of TGF-β processing affected the ability of GIC to induce Treg, we exposed PBMC to media derived from GIC treated with the PCSK inhibitor. This led to a decrease in the induction of the CD4+CD25+FOXP3+ population from 8.9 to 11.1 ± 0.5 % (Figure 15), indicating that the inhibition of TGF-β processing in GIC may reduce their immunosuppressive properties. Similar results were obtained for ZH-305 (data not shown).

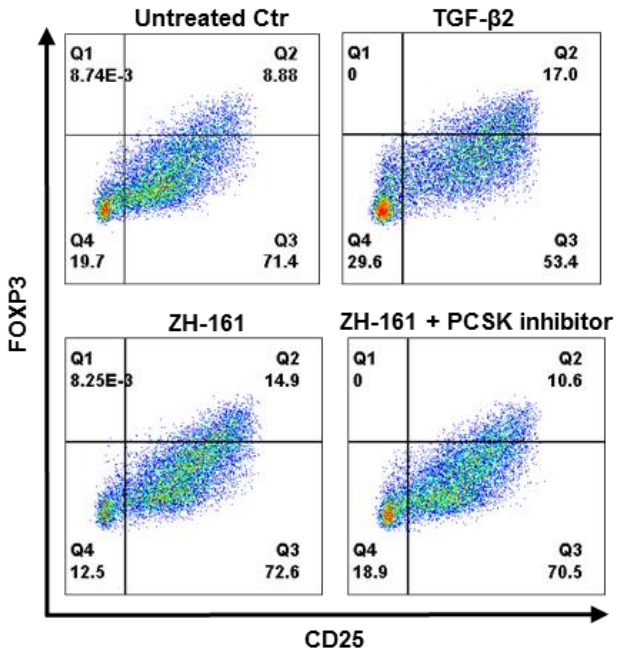

Figure 15. The inhibition of TGF-β processing in GIC reduces the immunosuppressive properties of GIC. Human PBMC were treated with 2 ng/ml TGF-β2 or conditioned media from untreated ZH-161 cells or ZH-161 cells treated with 25 µM PCSK inhibitor Decanoyl-RVKR-CMK, in the presence of IL-2, for 7 days. Cells were stained for CD4 (FITC), CD25 (APC) and FOXP3 (PE). Scatter plots are gated on CD4+. P=0.0185 for media of ZH-161 treated with PCSK-versus untreated ZH-161, unpaired Student's t-test.

Autocrine production of TGF-β may be necessary to maintain GIC stemness and TGF-β signaling in the tumor microenvironment (Pickup *et al.*, 2013), however, the mechanisms how GIC maintain high TGF-β activity is not well understood (Rodon *et al.*, 2014). We therefore evaluated the effect of TGF-β2 on furin levels. Indeed, TGF-β2 induced the expression of furin and FSA in all cell lines except T-269 (Figure 16A-C). To investigate whether the expression of PCSK other than furin was affected by TGF-β2, we analyzed the mRNA levels of the other six PCSK in ZH-161 (Figure 16D), T-325 and ZH-305 cells (data not shown) treated with TGF-β2. Indeed, none of them was changed, pointing towards a specific effect of TGF-β2 on furin expression. Concentration and time dependency of furin induction was investigated in ZH-161 cells (Figure 16E-G). Since we observed an induction on protein levels after 24 h (Figure 16F) and the maximum effect was achieved with 2.5 ng/ml TGF-β2 (Figure 16G), we selected these conditions for future experiments.

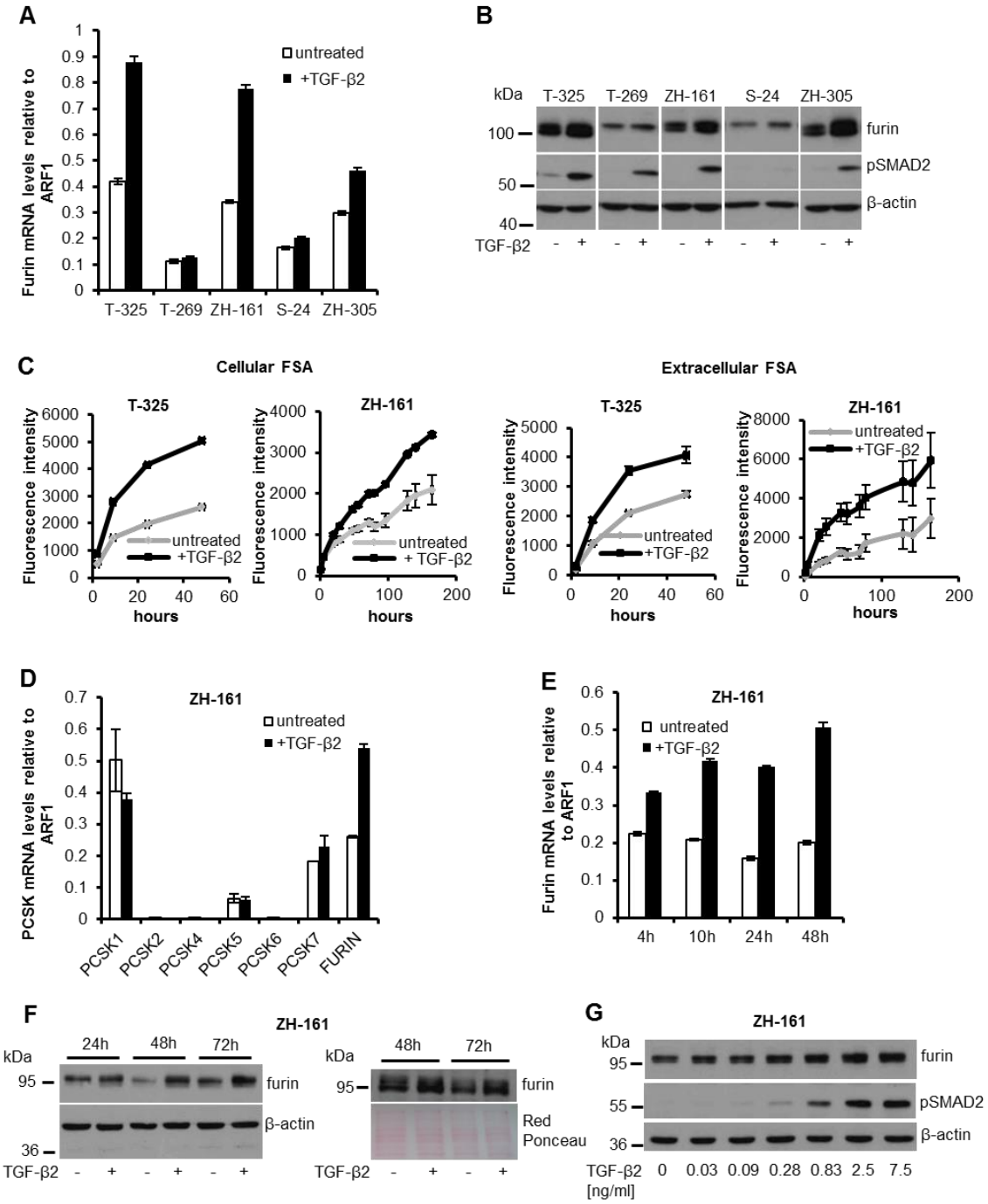

Figure 16. TGF-β2 induces furin in GIC. A-B. Furin expression was analyzed on mRNA level by RT-PCR (A) and on protein level in cell lysates by immunoblot (B) in GIC treated with 2.5 ng/ml TGF-β2 for 8 h (A) or 24 h (B). **C.** Furin-specific activity **(**FSA) was measured in the cell lysates and in conditioned media of T-325 and ZH-161 treated with 2.5 ng/ml TGF-β2 for 48 h. **D.** PCSK1, PCSK2, PCSK4, PCSK5, PCSK6, PCSK7 and furin mRNA levels in ZH-161 treated with 2.5 ng/ml TGF-β2 for 24 h. **E.** Furin mRNA levels were measured by RT-PCR in ZH-161 treated with TGF-β2 for the indicated time points. **F.** Furin protein levels were analyzed in cell lysates and conditioned media of ZH-161 treated with 2.5 ng/ml TGF-β2 for the indicated time points by immunoblot. **G.** ZH-161 were treated with the indicated concentrations of TGF-β2 for 24 h. Furin protein levels were analyzed in cell lysates by immunoblot.

We proceeded to study the signal transduction mechanisms involved in the regulation of furin by TGF-β2. Exposure of T-325, ZH-161 and ZH-305 to the ALK-5-specific inhibitor SD-208 had no effect on constitutive furin protein levels, but abolished TGF-β2-mediated furin induction (Figure 17). The transient gene silencing of SMAD2, SMAD3 or SMAD4 did not abrogate the induction of furin expression by TGF-β2, indicating involvement of SMAD-independent signal transduction (Figure 18).

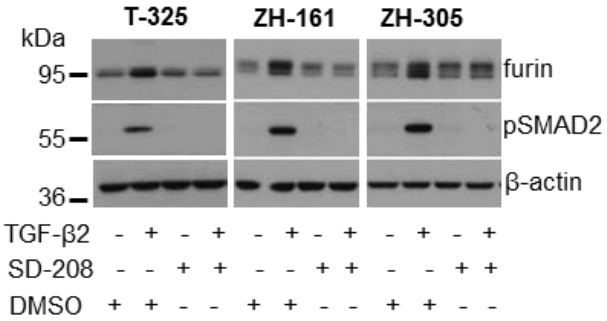

Figure 17. TGF-β2 induces furin in GIC in an ALK-5-dependent manner. Furin levels were analyzed in the lysates of T-325, ZH-161 and ZH-305 treated with 2.5 ng/ml TGF-β2, 1 μM SD-208 or the combination of TGF-β2 and SD-208 for 24 h. DMSO was used as solvent control.

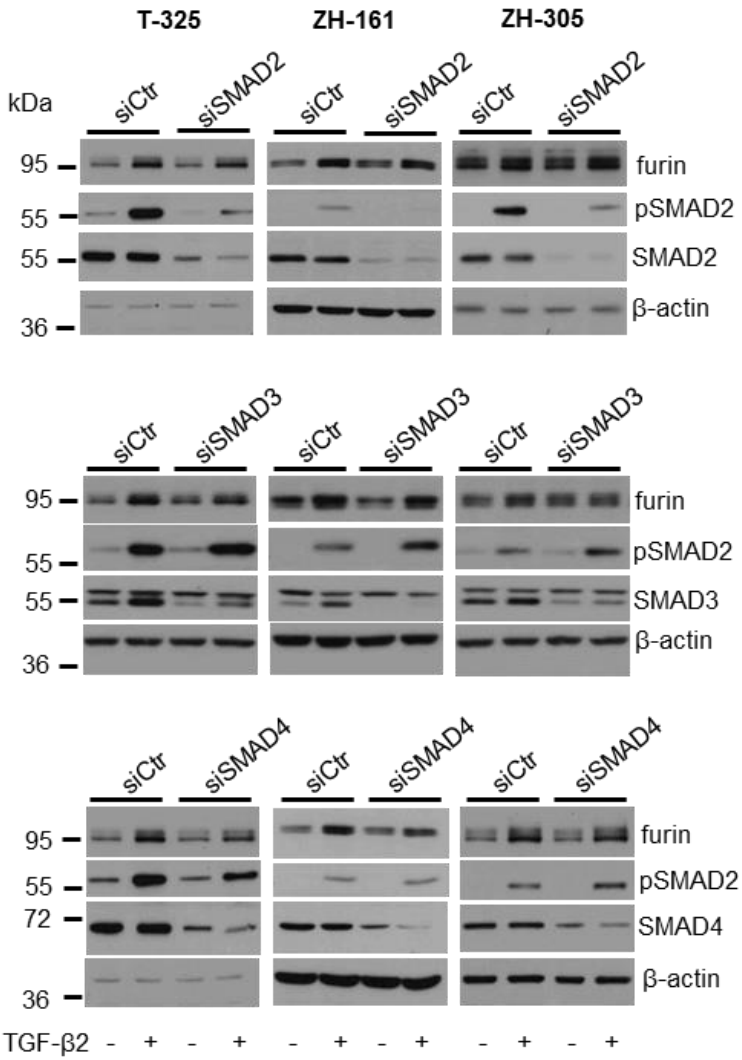

Figure 18. Furin induction by TGF-β2 in GIC is SMAD-independent. T-325, ZH-161 or ZH-305 were transfected with siRNA targeting SMAD2 (upper panel), SMAD3 (central panel), SMAD4 (lower panel) or non-targeting control and 24 h later treated with 2.5 ng/ml TGF-β2 for 24 h. Furin was analyzed in the cell lysates by immunoblot.

Indeed, blocking the MEK1/2 branch of the non-canonical TGF-β pathway by U0126 decreased constitutive furin levels and attenuated the increase in furin levels in response to TGF-β2. U0126 also decreased constitutive furin levels in T-269 cells which are the TGF-β2/furin-non-responsive model (Figure 19A). The inhibitory effect of U0126 on the induction of furin expression by TGF-β2 translated into a reduction in FSA (Figure 19B). Similarly, the selective gene silencing of ERK1, but not of ERK2, reduced furin basal levels in all cell lines and attenuated the induction of furin by TGF-β2 (Figure 20). Gene silencing of ERK1, but not of ERK2, reduced furin on mRNA level in both basal conditions and upon TGF-β2 treatment (Figure 21).

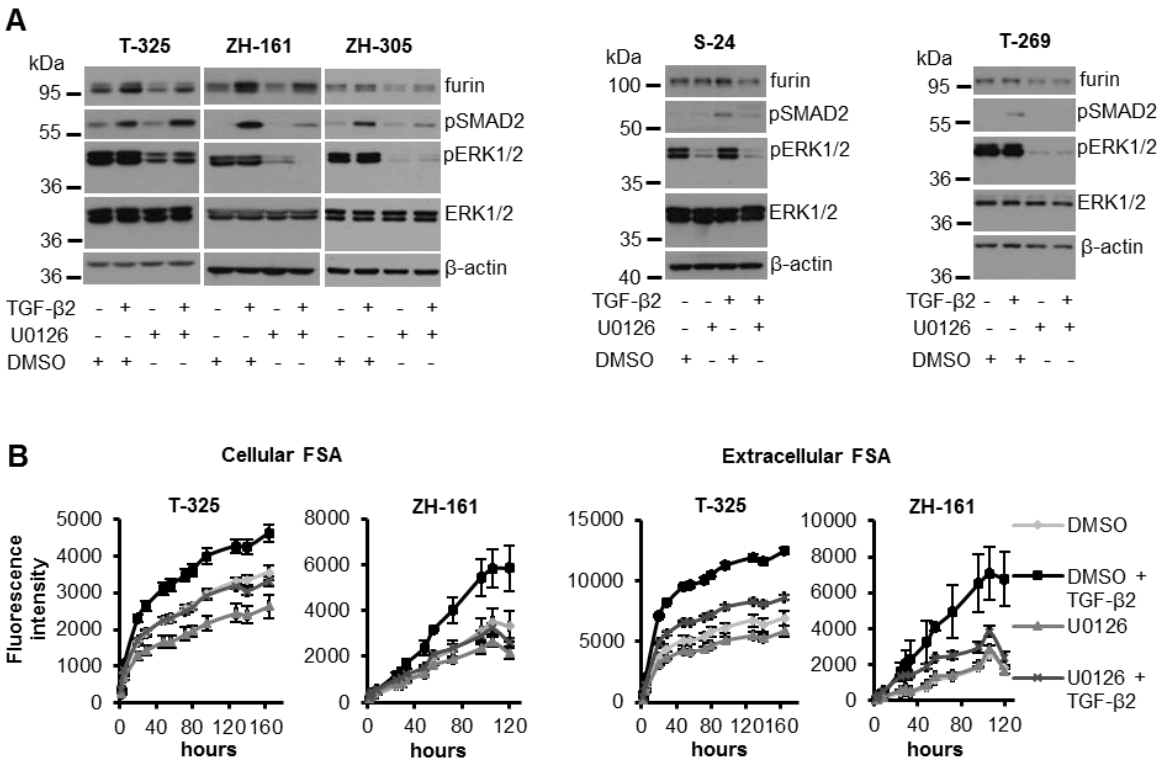

Figure 19. Furin induction by TGF-β2 requires the MEK1/2 pathway. A. Furin levels were determined in GIC pre-incubated with the MEK1/2 inhibitor U0126 (10 µM) for 1 h and then treated with 2.5 ng/ml TGF-β2 for 24 h. DMSO was used as solvent control. **B.** Furin-specific activity (FSA) was measured in the cell lysates and conditioned media of T-325 and ZH-161 cells pre-incubated with the MEK1/2 inhibitor U0126 (10 µM) or the respective DMSO control for 1 h and then treated with 2.5 ng/ml TGF-β2 for 48 h.

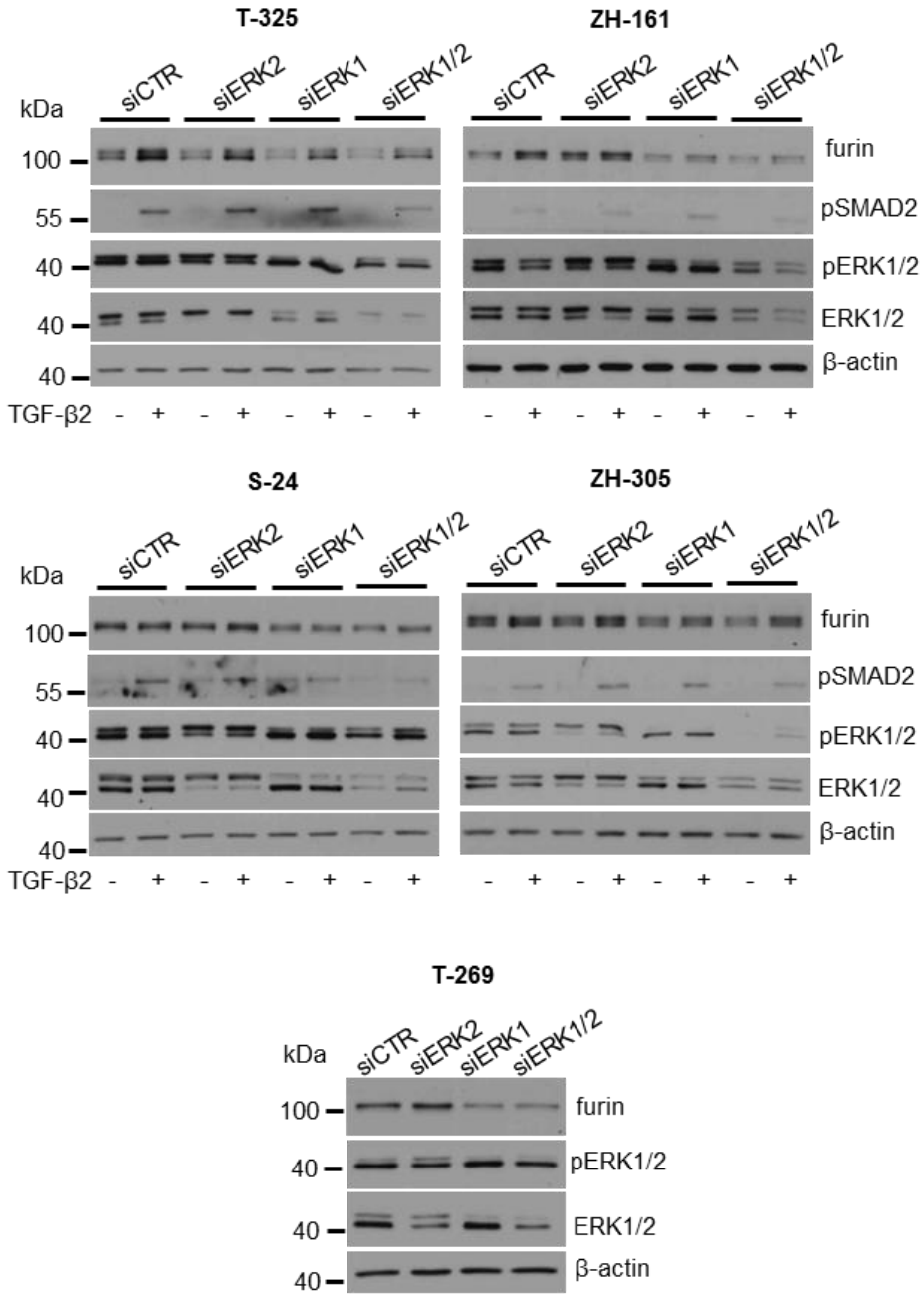

Figure 20. ERK1 controls basal and TGF-β2-induced furin levels. Cells were transfected with siRNA pools targeting ERK1, the combination of ERK1 and ERK2 or non-targeting control. 48 h later T-325, ZH-161, S-24 and ZH-305 were treated with TGF-β2 for 24 h. Furin was analyzed in the cell lysates by immunoblot.

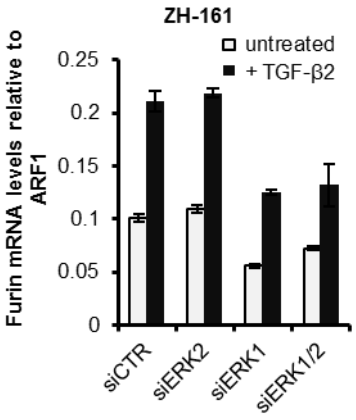

Figure 21. ERK1 controls furin mRNA levels. Furin mRNA levels were determined by RT-PCR in ZH-161 cells transfected with siRNA targeting ERK1, ERK2, the combination of ERK1 and ERK2 or non-targeting control siRNA and 24 h later treated with TGF-β2 for 24h. Data are the mean of triplicates ± SD.

To address whether the control of furin by ERK1 and TGF-β2 is associated with a glioma stem cell-like phenotype, we examined the same GIC cultured in differentiating conditions (Figure 22A) and the long-term glioma cell lines, LN-308 and LN-229 (Figure 22B). In both cases ERK1 gene silencing reduced basal furin levels and attenuated the induction of furin by TGF-β2, suggesting that this molecular pattern is shared between cells with stemness properties and more differentiated tumor cells.

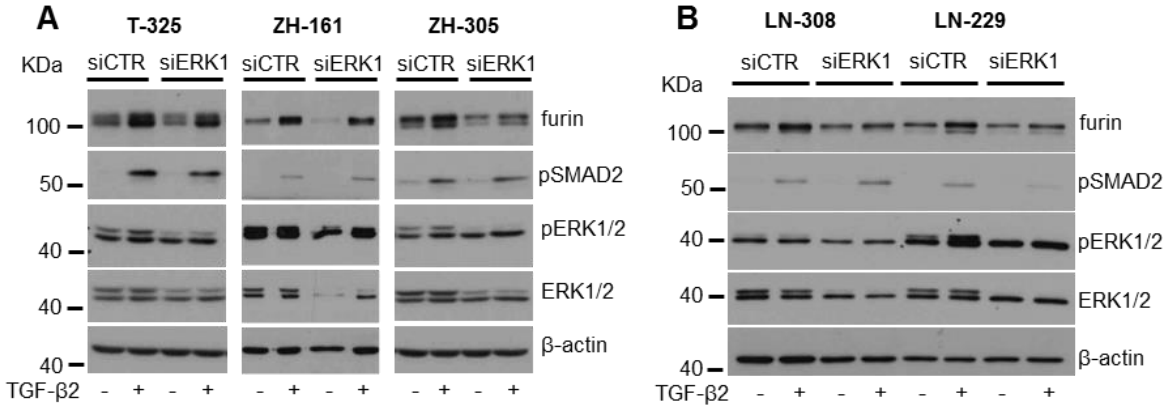

Figure 22. TGF-β and ERK1 control furin levels in GIC cultured in differentiated conditions and in long-term glioma cell lines. A. T-325, ZH-161 and ZH-305 cells were cultured in NBM containing 5% FCS for 7 days. Cells were then transfected with siRNA targeting ERK1 and 48 h later treated with TGF-β2 for 24 h. Furin was analysed in the cell lysates by immunoblot. **B.** LN-229 and LN-308 cells were transfected with siRNA pools targeting ERK1 or non-targeting control. 48 h later cells were treated with TGF-β2 for 24 h. Furin was analysed in the cell lysates by immunoblot.

The reduction in furin levels following ERK1 gene silencing was associated with a reduction in pro-TGF-β1/2 processing with increased pro-TGF-β levels and decreased mature TGF-β levels (Figure 23). MEK1/2 inhibition did not significantly affect the number of spheres, but the spheres formed by GIC (T-325, ZH-161 and ZH-305) treated with U0126 were smaller, suggesting an inhibitory effect on cell proliferation as confirmed by reduced MTT metabolism (Figure 24).

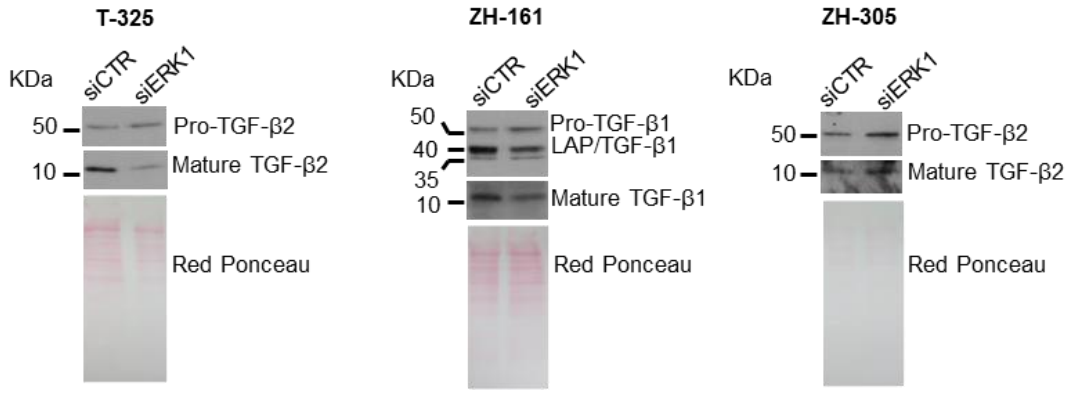

Figure 23. ERK1 gene silencing leads to reduced TGF-β processing in GIC. T-325, ZH-161 and ZH-305 cells were transfected with siRNA targeting ERK1. 24 h later cells were put in fresh media and further incubated for 72 h. Precursor and mature forms of TGF-β1 and TGF-β2 were then determined by immunoblot in the conditioned media.

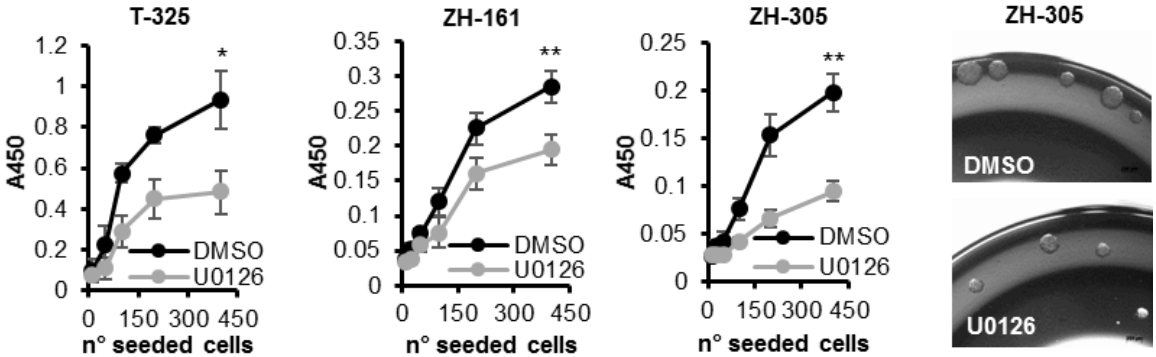

Figure 24. MEK1/2 inhibition reduces proliferation of GIC. Sphere formation assay in T-325, ZH-161 and ZH-305 cells treated with 10 µM U0126 or solvent control for 15 days. Data are absorbance values after MTT addition (* p<0.05, **p<0.01, unpaired Student's t-test).

Targeting ERK1/2 also attenuated the immunosuppressive properties of GIC since treatment of ZH-161 and ZH-305 cells with U0126 decreased the induction of

CD4+CD25+FOXP3+ cells by conditioned media of ZH-161 (Figure 25) and ZH-305 (data not shown).

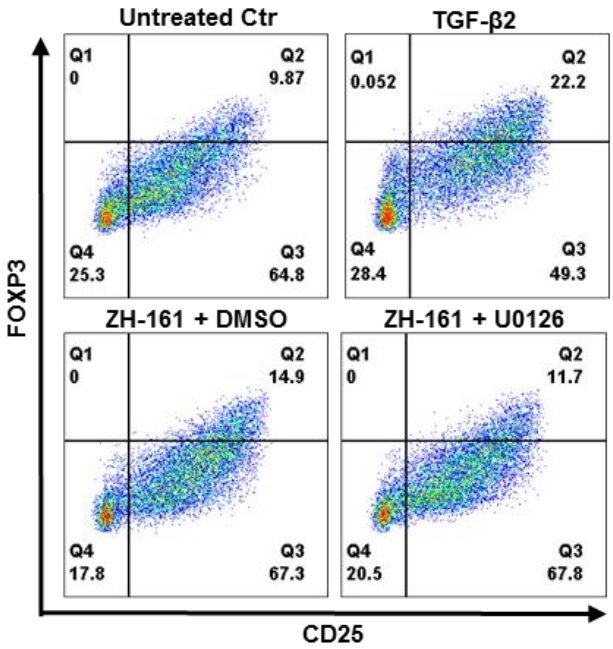

Figure 25. MEK1/2 inhibition reduces immunosuppressive properties of GIC. Human PBMC were treated with conditioned media from ZH-161 treated with U0126 or DMSO. Cells were stained for CD4 (FITC), CD25 (APC) and FOXP3 (PE). Scatter plots are gated on CD4+. P=0.0024, unpaired Student's t-test, for media of ZH-161 treated with U0126-versus ZH-161 treated with DMSO.

6.1.2. Discussion

Glioblastoma is the most aggressive brain tumor in adults. Conventional glioblastoma treatment is based on tumor surgical resection, followed by radio-chemotherapy. Despite current multimodal therapies, glioblastomas relapse and the prognosis is poor (Reifenberger *et al.*, 2016). GIC have been attributed an important role in tumor initiation, therapy resistance and recurrence. In addition, immunosuppressive properties due to cell-to-cell interactions as well as due to the secretion of immunosuppressive cytokines, such as TGF-β, have been ascribed to GIC (Lathia *et al.*, 2015). Indeed, TGF-β is a key cytokine in glioblastoma biology since it controls several hallmarks of the disease, including the tumorigenic activity of GIC (Joseph *et al.*, 2013). GIC express high levels of TGF-β and TGF-β has been shown to promote maintenance of stemness properties of GIC (Ikushima *et al.*, 2009). Due to the role played in the malignant phenotype of glioblastoma, TGF-β has attracted attention as a potential target for glioblastoma treatment. Different approaches have been pursued to inhibit TGF-β, with the development of TGF-β oligonucleotides (Hau *et al.*, 2007), anti-TGF-β antibodies (den Hollander *et al.*, 2015) and inhibitors of TGF-β signaling (Han *et al.*, 2015). TGF-β signal transduction is mainly targeted using TGF-β receptor inhibitors such as SD-208 (Uhl *et al.*, 2004). In this work we pursued an alternative approach focusing on TGF-β processing and investigating the role played by PCSK in TGF-β processing in GIC. Among the PCSK candidates we identified furin as an extracellular regulator of TGF-β activity in GIC. Indeed, we demonstrated that furin is the main enzyme involved in TGF-β processing in GIC and that this process may take place to a greater extent extracellularly (Figure 12). These data thus allowed the identification of furin as a potential extracellular target to counteract TGF-β in gliomas.

Furin and the other PCSK are deregulated in several pathological conditions, including cancer (Seidah and Prat, 2012). For this reason, PCSK have been considered as potential therapeutic targets in the past and several inhibitors have been generated so far. However, their use is limited by the lack of the strict specificity of the currently available molecules for the individual PCSK (Seidah and Prat, 2012). The use of oligonucleotides specific for furin may represent an alternative therapeutic strategy allowing to overcome the specificity issue. Recently, a phase I clinical trial based on DNA/autologous tumor cell vaccination in

combination with furin gene-silencing brought promising results (Senzer *et al.*, 2012). Another limitation of the therapeutic inhibition of furin as well as of some other PCSK is their ubiquitous expression. Beyond, furin processes a broad range of substrates which are essential for tissue homeostasis. Thus, inhibition of furin, in particular in case this is done in a systemic way, may lead to severe side effects on normal tissues. Thus, targeted strategies or local administration of the inhibitors/oligonucleotides are required. Interestingly according to the work presented here, furin, which was meant to act mainly intracellularly in the trans-Golgi network (Thomas, 2002), acts to a greater extent extracellularly in the case of TGF-β processing in GIC. Thus, the development of furin blocking agents acting only extracellularly, such as cell non-permeable inhibitors or antibodies, may potentially represent a strategy to partially overcome the inhibition of the processing of other substrates than TGF-β.

GIC express high levels of TGF-β. Autocrine production of TGF-β may be necessary to maintain GIC stemness and TGF-β signaling in the tumor microenvironment. Previous works suggested that in glioma cells TGF-β promotes its expression in an autocrine positive loop (Rodon *et al.*, 2014). In this work, we uncovered the existence of an additional mechanism by which TGF-β sustains its activity, involving furin. Indeed, this work demonstrates a positive feedback loop where TGF-β promotes furin expression in GIC (Figure 16). Notably, the control of furin expression by TGF-β does not involve the canonical/SMAD-dependent signaling pathway (Figure 18), but it relies on the MEK/ERK pathway (Figure 19). Specifically, our results suggest that ERK1 controls basal furin levels in GIC and mediates the induction of furin by TGF-β2 (Figure 20). Importantly, the inhibition of the MEK1/2 pathway translated into a reduction of TGF-β processing and decreased proliferation of GIC (Figure 23-24).

The MEK/ERK pathway is a key regulatory signaling pathway involved in the control of cell survival, proliferation and differentiation. The MEK/ERK cascade is often deregulated in cancers, mainly as a consequence of mutations affecting the up-stream regulators Raf serine/threonine kinases and RAS small GTPase (Zhao and Adjei, 2014). Also, since the ERK signaling acts downstream to receptors tyrosine kinases such as EGFR, ERK activation is increased when receptor tyrosine kinases are overexpressed or more active due to mutations (Zhao and Adjei, 2014). In glioblastoma, several mutations affect the receptor tyrosine kinase/RAS/Raf

signaling pathway and overexpression or mutation of EGFR are common (Verhaak *et al.*, 2010; Reifenberger *et al.*, 2016). Indeed, ERK activity is up-regulated in glioblastoma (Lopez-Gines *et al.*, 2008). Because of its aberrant regulation in cancers, the ERK pathway has attracted attention as a potential therapeutic target, with the consequent development of different MEK1/2 pharmacological inhibitors, such as selumetinib, trametinib and binimetinib, which are at present under clinical investigation in cancer patients (Zhao and Adjei, 2014). Our data suggest that ERK1 inhibition in GIC may also affect TGF-β levels by attenuating furin expression. Thus, the specific block of ERK1 signaling may inhibit the autocrine loop by which TGF-β promotes its processing. In conclusion, due to the elucidation of the autocrine loop by which TGF-β sustains its expression and processing in GIC, involving furin and ERK1, this work may be useful to develop more sophisticated therapeutic strategies to counteract high TGF-β activity in glioblastoma.

6.2 Transforming growth factor (TGF)-β induces the splice variants extra-domain A (EDA) and extra-domain B (EDB) of fibronectin to control TGF-β superfamily activity in endothelial cells

A related manuscript is in preparation.

Experimental contribution: I performed all the experiments of this work under the supervision of Isabel Burghardt and Michael Weller. Antibodies to fibronectin and fibronectin recombinant fragments were provided by Luciano Zardi.

6.2.1 Results

6.2.1.1 TGF-β1 and TGF-β2 induce FN, EDA+FN and EDB+FN expression in endothelial cells.

We first investigated the expression of total FN and of the FN isoforms containing the extra-domains A and B in ten human glioblastoma sections. We used the antibodies IST4, reacting with a FN domain common to all FN isoforms, IST9, reacting with the EDA domain, and C6, specific for EDB+FN (Figure 26). Tumor-associated vessels showed high levels of FN and of the two isoforms EDA+FN and EDB+FN, whereas the bulk tumor was negative (Figure 27). Accordingly, an analysis of freshly dissociated glioblastoma samples showed that FN, EDA+FN and EDB+FN were specifically expressed in the CD31+ cell fraction (Figure 28).

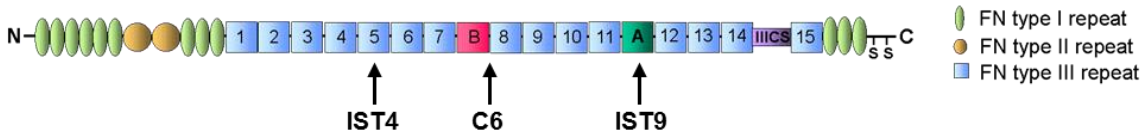

Figure 26. Schematic representation of the modular structure of FN. The target regions of the monoclonal antibodies specific for the FN type III repeats 5 (IST-4), EDA+FN (IST-9) and EDB+FN (C6) are indicated.

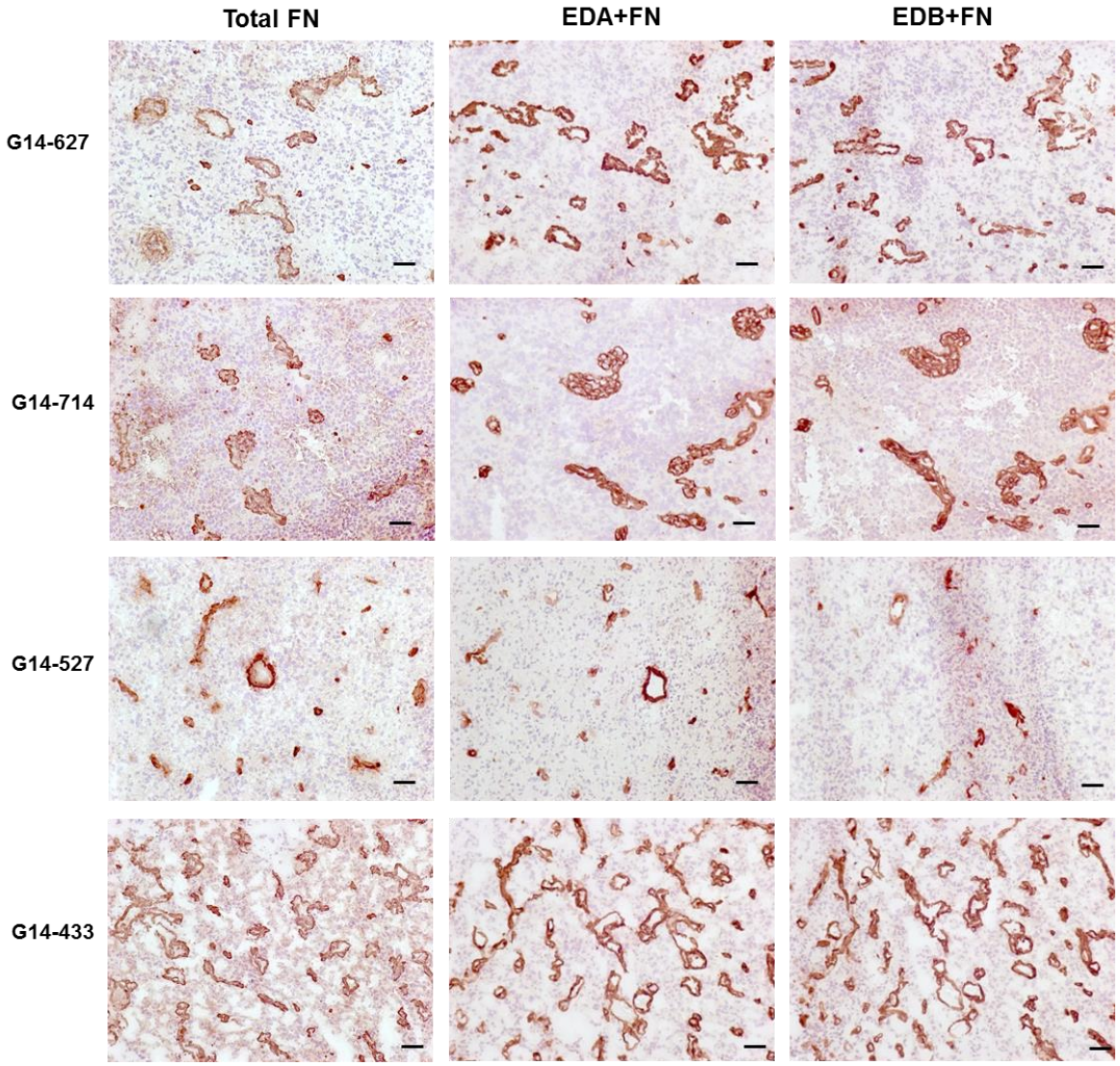

Figure 27. EDA+FN and EDB+FN are expressed in glioblastoma tumor vasculature. Total FN, EDA+FN and EDB+FN were immunohistochemically detected in frozen glioblastoma sections. Bars: 50 µm.

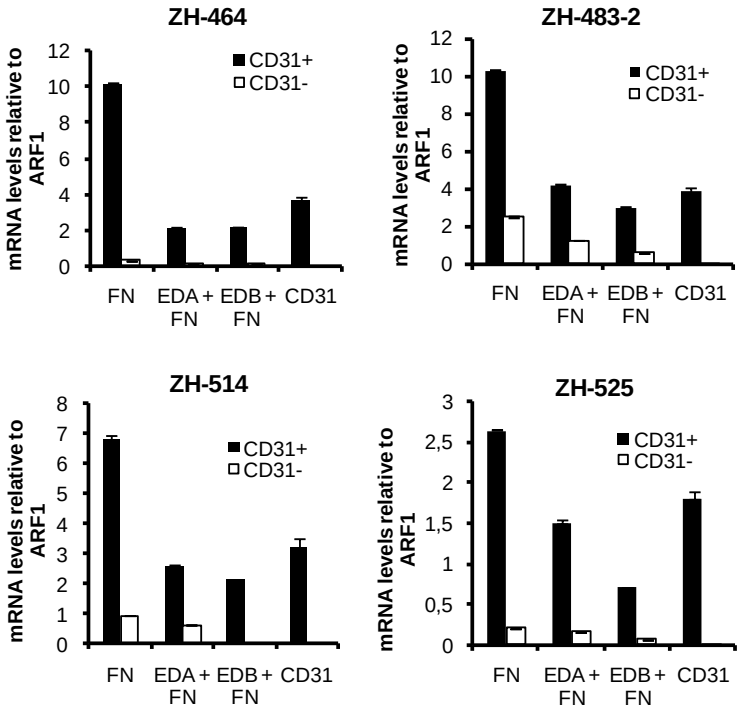

Figure 28. FN, EDA+FN and EDB+FN are preferentially expressed in CD31+ cells in glioblastoma. Total FN, EDA+FN and EDB+FN mRNA levels were determined in CD31+ *versus* CD31- cells isolated from freshly dissociated glioblastoma tissues by RT-PCR.

Considering the vascular localization of FN and its isoforms in glioblastoma we aimed at investigating the endothelial compartment in appropriate *in vitro* cell models. As alternative splicing of FN respectively the generation of EDA+FN and EDB+FN is mainly regulated by TGF-β in several cell types, we used human brain-derived microvascular endothelial cells (hCMEC) to investigate the effect of TGF-β1 and TGF-β2 treatment on FN, EDA+FN and EDB+FN levels. Indeed, treatment with both TGF-β1 and TGF-β2 increased mRNA and protein levels of total FN, EDA+FN and EDB+FN (Figure 29A). In order to decipher the molecular mechanism by which TGF-β1 and TGF-β2 induce FN expression in hCMEC, we first used the ALK-5-specific inhibitor SD-208. SD-208 abolished the TGF-β1- and TGF-β2-mediated induction of FN, EDA+FN and EDB+FN on both mRNA and protein level (Figure 29A), indicating that the process is ALK-5-dependent and equally valid for TGF-β1 and TGF-β2. To further specify the signal transduction pathway downstream to TGF-β receptors, we first investigated the potential involvement of the SMAD signaling pathway. Gene silencing of SMAD4 inhibited the TGF-β1- and TGF-β2-induced increase in FN, EDA+FN and EDB+FN on mRNA and protein levels,

indicating the involvement of the SMAD signaling pathway (Figure 29B, data for TGF-β1 not shown). In order to investigate the R-SMAD involved in the process, we performed gene silencing of SMAD2 and SMAD3. Gene silencing of SMAD3, but not of SMAD2 (Figure 29C, left upper panel), abolished the TGF-β1- and TGF-β2-dependent induction of FN, EDA+FN and EDB+FN on mRNA level (data for TGF-β1 not shown). In addition, gene silencing of SMAD3 reduced the relative fraction of FN molecules containing the EDB domain by two-fold, whereas the relative fraction of FN molecules containing EDA remained unchanged (Figure 29C, upper panels). These data suggest a specific role for SMAD3 in the control of the splicing of the EDB domain. The inhibitory effect of SMAD3 gene silencing on TGF-β1- and TGF-β2-mediated induction of FN, EDA+FN and EDB+FN was confirmed on protein levels (Fig. 29C, lower panels, data for TGF-β1 not shown).

Since a role for JNK in the TGF-β-dependent induction of FN expression has been reported in human fibrosarcoma cells (Hocevar *et al.*, 1999) and in light of the known cross-talk between canonical SMAD-dependent and non-canonical SMAD-independent TGF-β signaling pathways, we also explored the potential role of JNK in our model. The JNK-specific inhibitor SP600125 did not influence TGF-β2-dependent induction of total FN or EDA+FN, but it abolished the TGF-β2-dependent induction of EDB+FN on mRNA and protein levels (Figure 30). These data suggest that the JNK signaling pathway is involved in the control of EDB splicing in hCMEC cells. Inhibition of the other branches of the non-canonical TGF-β signaling cascade using specific inhibitors for p38, MEK1/2 and AKT neither affected FN on basal level nor its TGF-β2-dependent induction (data not shown), pointing towards a specific role of JNK in our model.

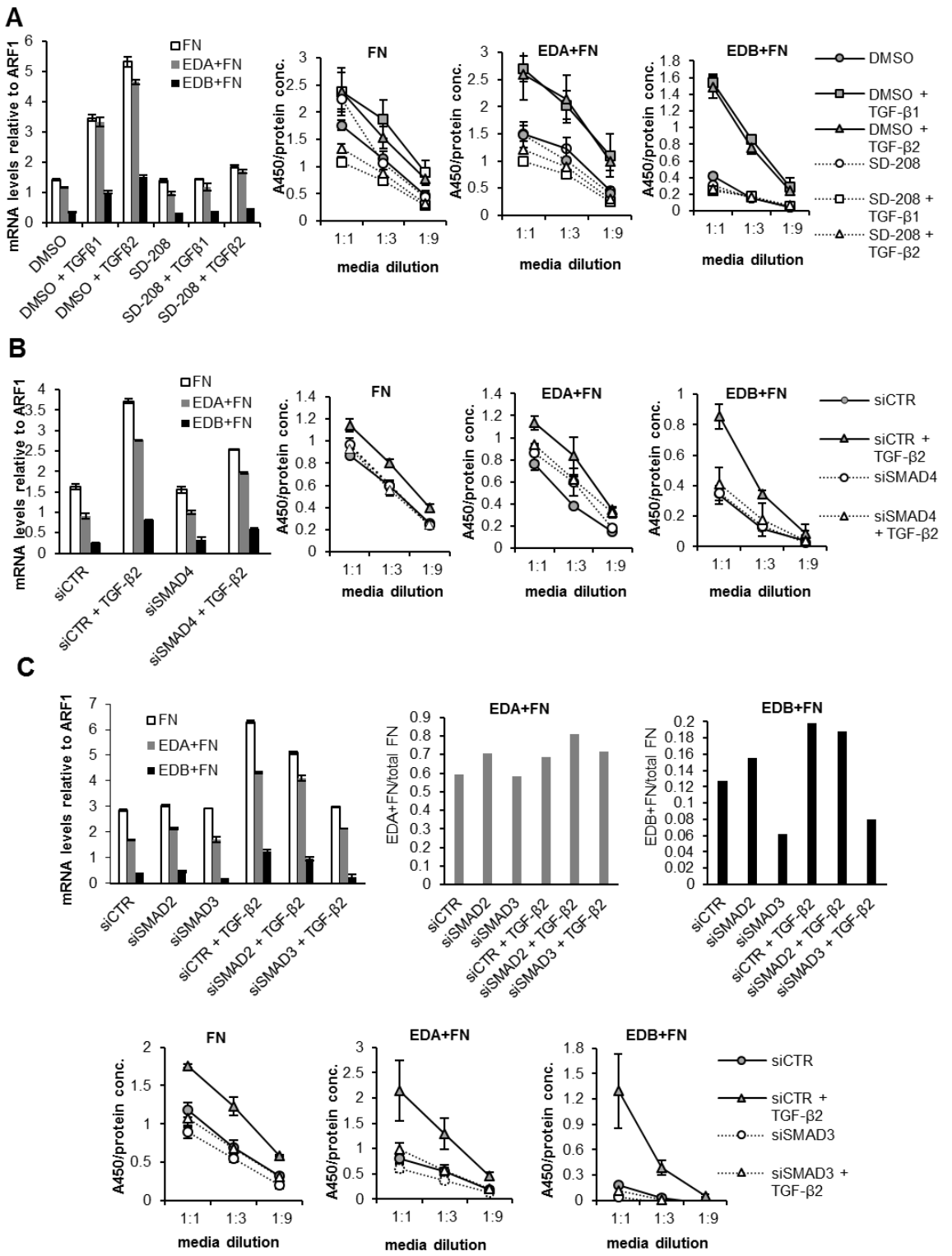

Figure 29. TGF-β1 and TGF-β2 induce EDA+/EDB+FN expression in hCMEC in an ALK5- and SMAD3/SMAD4-dependent manner. In all experiments FN, EDA+FN and EDB+FN levels were determined 48 h post TGF-β treatment on mRNA level by RT-PCR and 72 h post TGF-β treatment on protein level by ELISA. Data are expressed as means of triplicates ± SD. **A.** FN, EDA+FN and EDB+FN mRNA (left panel) and protein levels (other panels) in hCMEC cells incubated with 1 µM SD-208 or DMSO as solvent control for 1 h and then treated with 5 ng/ml TGF-β1 or TGF-β2. **B.** FN,

EDA+FN and EDB+FN mRNA (left panel) and protein (other panels) levels in hCMEC transfected with siRNA targeting SMAD4 or non-targeting control and treated with 5 ng/ml TGF-β2 24 h post-transfection. **C.** FN, EDA+FN and EDB+FN mRNA levels (left upper panel) in hCMEC transfected with siRNA targeting SMAD2, SMAD3 or non-targeting control and treated with 5 ng/ml TGF-β2 24 h post-transfection. The fraction of FN molecules containing the EDA domain (EDA+FN/total FN) or EDB domain (EDB+FN/total FN), using the RT-PCR data reported on the left panel, are indicated. FN, EDA+FN and EDB+FN protein levels in hCMEC transfected with siRNA targeting SMAD3 or non-targeting control and treated with 5 ng/ml TGF-β2 24 h post-transfection (lower panels).

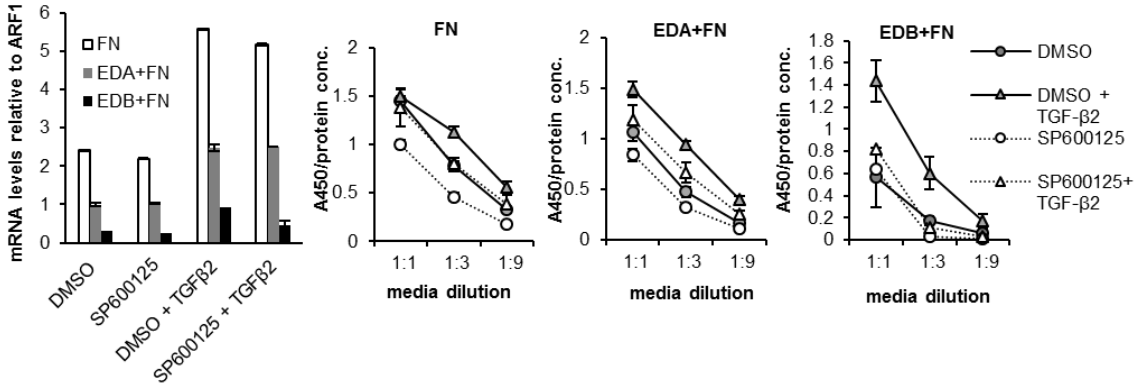

Figure 30. TGF-β2-mediated induction of EDB+FN involves the JNK pathway. FN, EDA+FN and EDB+FN mRNA (left panel) and protein levels (other panels) in hCMEC pre-incubated with 10 μM SP600125 for 2 h and then treated with 5 ng/ml TGF-β2 for 48 h (left panel) or 72 h (other panels).

In order to investigate this process in a model closely reflecting the *in vivo* situation, we analyzed the effect of TGF-β1 and TGF-β2 on FN in human glioblastoma-derived endothelial cells (GMEC) derived from freshly dissociated tumor tissue by isolating CD31+ cells. TGF-β2 induced the expression of FN, EDA+FN and EDB+FN in the two patient-derived cell lines ZH-459 CD31+ and ZH-613 CD31+ (Figure 31A, B). We then investigated the molecular mechanism in the cell line ZH-613. In line with our results for hCMEC (Figure 29), TGF-β2-induced expression of FN, EDA+FN and EDB+FN was ALK-5-dependent (Figure 31B) and SMAD3/SMAD4-dependent (Figure 31C). In CD31+ cells derived from another glioblastoma patient (ZH-616), TGF-β2 did not induce FN, EDA+FN and EDB+FN, but the gene silencing of SMAD3 and SMAD4 reduced the basal levels of EDA+FN and EDB+FN (Figure 31D).

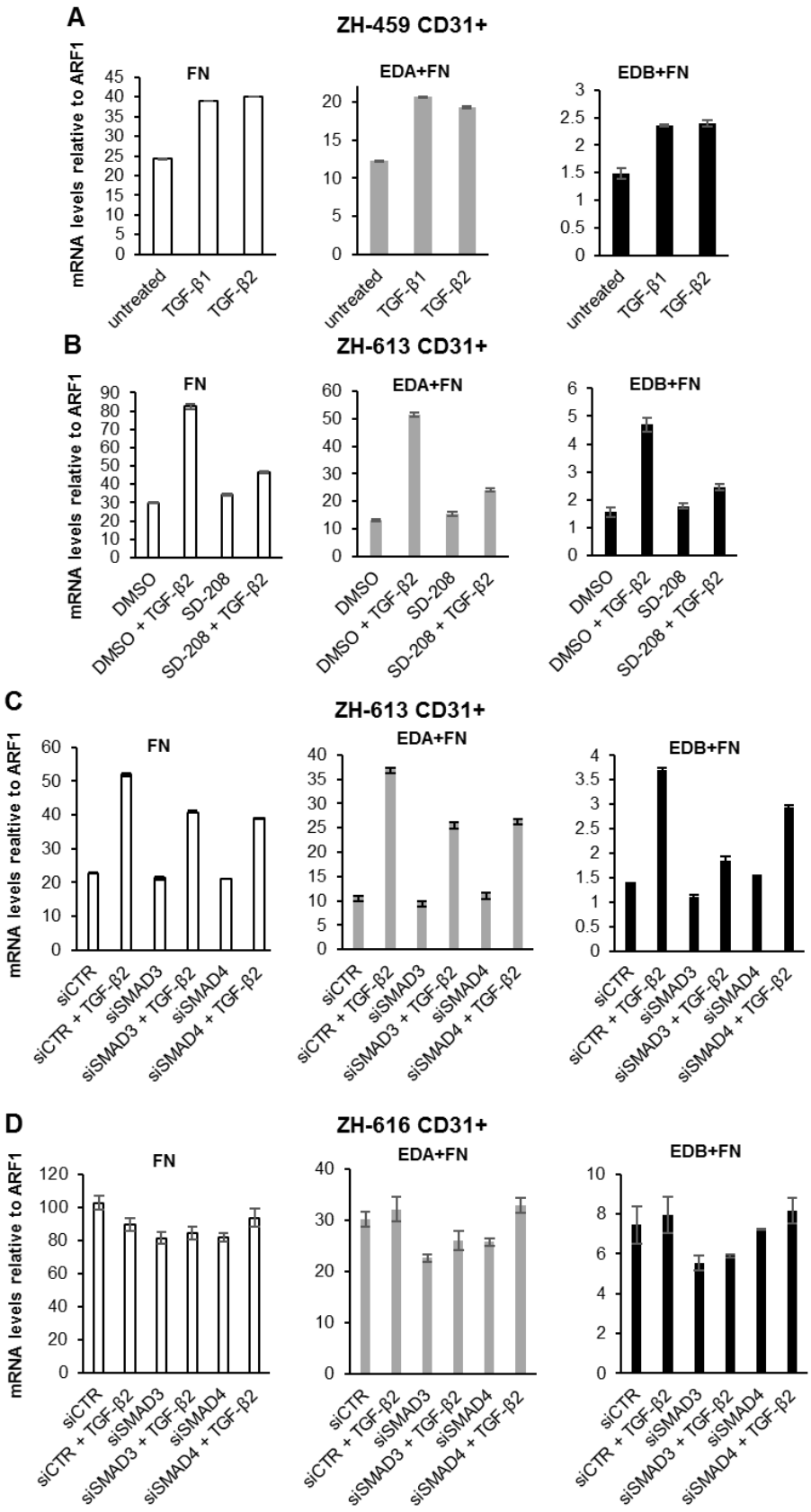

Figure 31. TGF-β1 and TGF-β2 induce EDA+/EDB+FN expression in CD31+ cells derived from glioblastoma in a ALK-5- and SMAD4/SMAD3-dependent manner. A. FN, EDA+FN and EDB+FN mRNA levels in ZH-459 CD31+ cells treated with 5 ng/ml TGF-β1 or TGF-β2 for 48 h. **B.** FN, EDA+FN

and EDB+FN mRNA levels in ZH-613 CD31+ cells incubated for 1 h with 1 µM SD-208 or DMSO as solvent control and then treated with 5 ng/ml TGF-β2 for 48 h. **C.** FN, EDA+FN and EDB+FN mRNA levels in ZH-613 CD31+ cells transfected with siRNA targeting SMAD3, SMAD4 or non targeting control and then treated with 5 ng/ml TGF-β2 24 h post-transfection for 48 h. **D.** FN, EDA+FN and EDB+FN mRNA levels in ZH-616 CD31+ cells transfected with siRNA targeting SMAD3, SMAD4 or non targeting control and then treated with 5 ng/ml TGF-β2 24 h post-transfection for 48 h. Data are mean of triplicates ± SD.

6.2.2.2 EDA+FN and EDB+FN promote TGF-β superfamily/SMAD1,5 signaling in hCMEC.

To investigate the potential control of TGF-β signaling by EDA+FN and EDB+FN in endothelial cells, we performed transient gene silencing of EDA+FN or EDB+FN in hCMEC (Figure 32A, left panel) and checked for phosphorylation levels of R-SMAD (Figure 32A). The levels of pSMAD1,5 were reduced in both siEDA+FN and siEDB+FN gene-silenced cells. Cells with EDA+FN gene silencing showed also reduced leves of pSMAD3. The levels of pSMAD2 were unaffected (Figure 32A). To evaluate the signaling cascade further downstream, we determined mRNA levels of ID-1 and PAI-1 as downstream targets of the putative ALK-1/pSMAD1,5,8 and ALK-5/pSMAD2,3 branches, respectively. The gene silencing of EDA+FN and EDB+FN decreased ID-1 and increased PAI-1 expression levels in hCMEC cells (Figure 32B). Since the gene silencing of EDA+FN also affected total levels of FN and EDB+FN levels (see Figure 32A, left panel), to confirm the specific involvement of the EDA and EDB domains, we used a different approach, based on the use of recombinant FN fragments including or excluding the EDA and EDB domains (Figure 33A). HCMEC cells treated with a recombinant FN fragment containing the EDB domain and adjacent FN type III domains 7, 8 and 9 (7-EDB-8-9) showed reduced levels of pSMAD1,5 compared with untreated cells or cells treated with the corresponding FN fragment lacking the EDB domain (7-8-9). The levels of pSMAD2 and pSMAD3 were unchanged (Figure 33B). On mRNA levels the treatment with 7-EDB-8-9 decreased the levels of ID-1 and increased the levels of PAI-1 (Figure 33C). Treatment with the recombinant EDB domain alone (without the adjacent domains 7, 8 and 9) had only minor effects on either pSMAD1,5 and ID-1 or PAI-1 levels, indicating the involvement of flanking domains in mediating the EDB effect. Compared to untreated cells or cells treated with the recombinant fragments

including the two flanking EDA domains 11-12 and lacking EDA, hCMEC cells treated with the recombinant EDA domain alone showed decreased levels of pSMAD1,5 whereas the levels of pSMAD2 and pSMAD3 were unaffected. pSMAD1,5 levels were reduced in cells treated with the FN fragment 11-12 to a lesser extent than in cells treated with the EDA domain alone (Figure 33B). On mRNA level, EDA treatment reduced the levels of ID1 and increased the levels of PAI-1 (Figure 33C). These data suggest that both the EDA and the EDB domains of FN are involved in the control of TGF-β superfamily signaling in hCMEC cells.

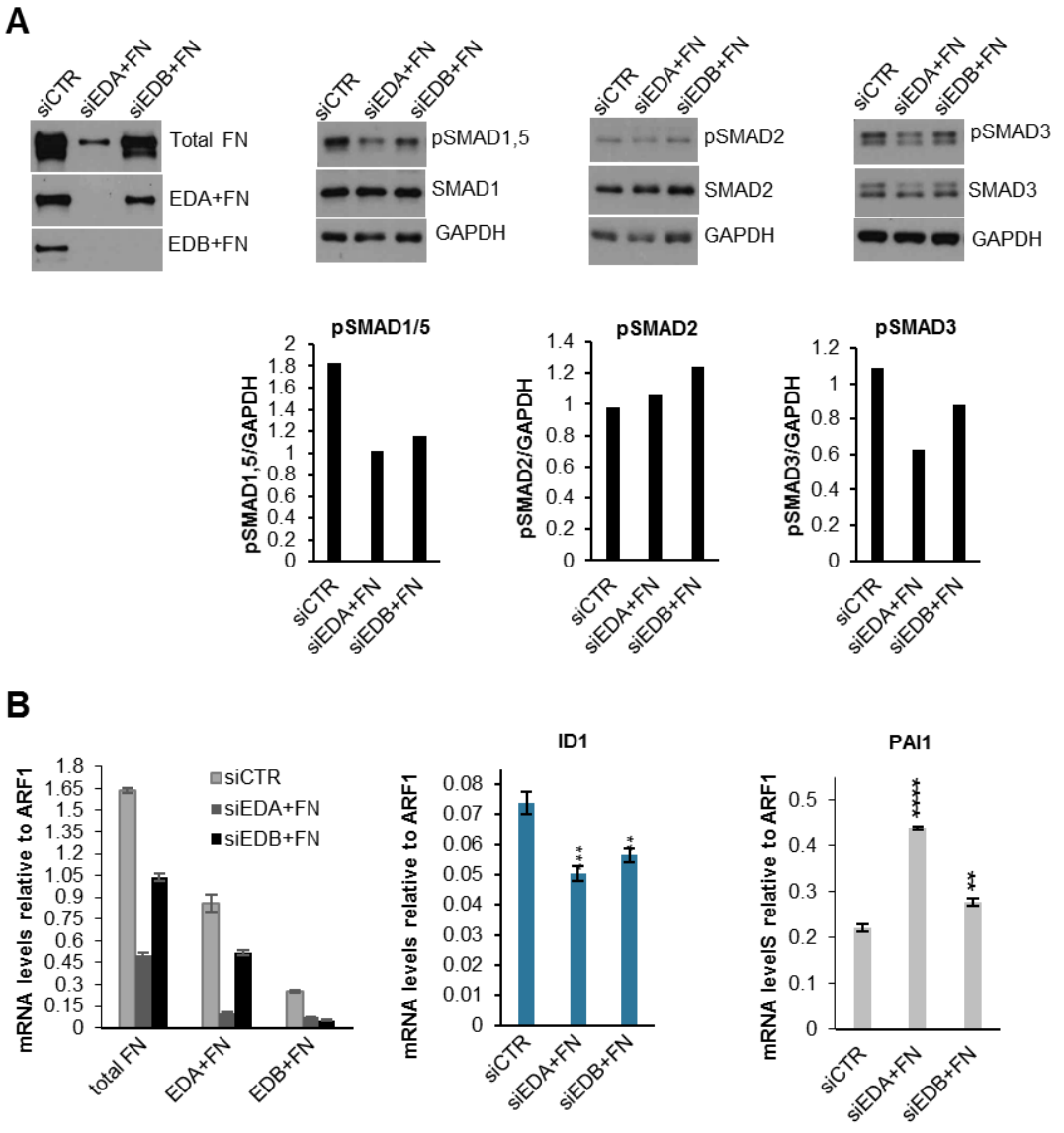

Figure 32. The gene silencing of EDA+FN and EDB+FN in hCMEC decreases SMAD1,5 phosphorylation. A. hCMEC cells were transfected with siRNA targeting the EDA or EDB domain of FN or non-targeting control siRNA. At 24 h post transfection cells were transferred to a new dish and cultured in full medium. At 48 h post transfection cells were put in serum-free medium for 48 h.

Concentrated conditioned culture media were analyzed for the levels of total FN, EDA+FN and EDB+FN (upper left panel). The levels of phosphorylated and total SMAD1,5, SMAD2 and SMAD3 were analyzed in cell lysates. The densitometry analysis of the immunoblot is indicated. **B.** FN, EDA+FN, EDB+FN, ID-1 and PAI-1 mRNA levels in hCMEC cells were determined by RT-PCR following gene silencing of EDA+FN or EDB+FN and compared to non-targeting control. Data are expressed as mean ± SD (***P*<.01, ****P*<.001, *****P*<.0001, one-way Anova followed by Tukey's post hoc test 95% CI).

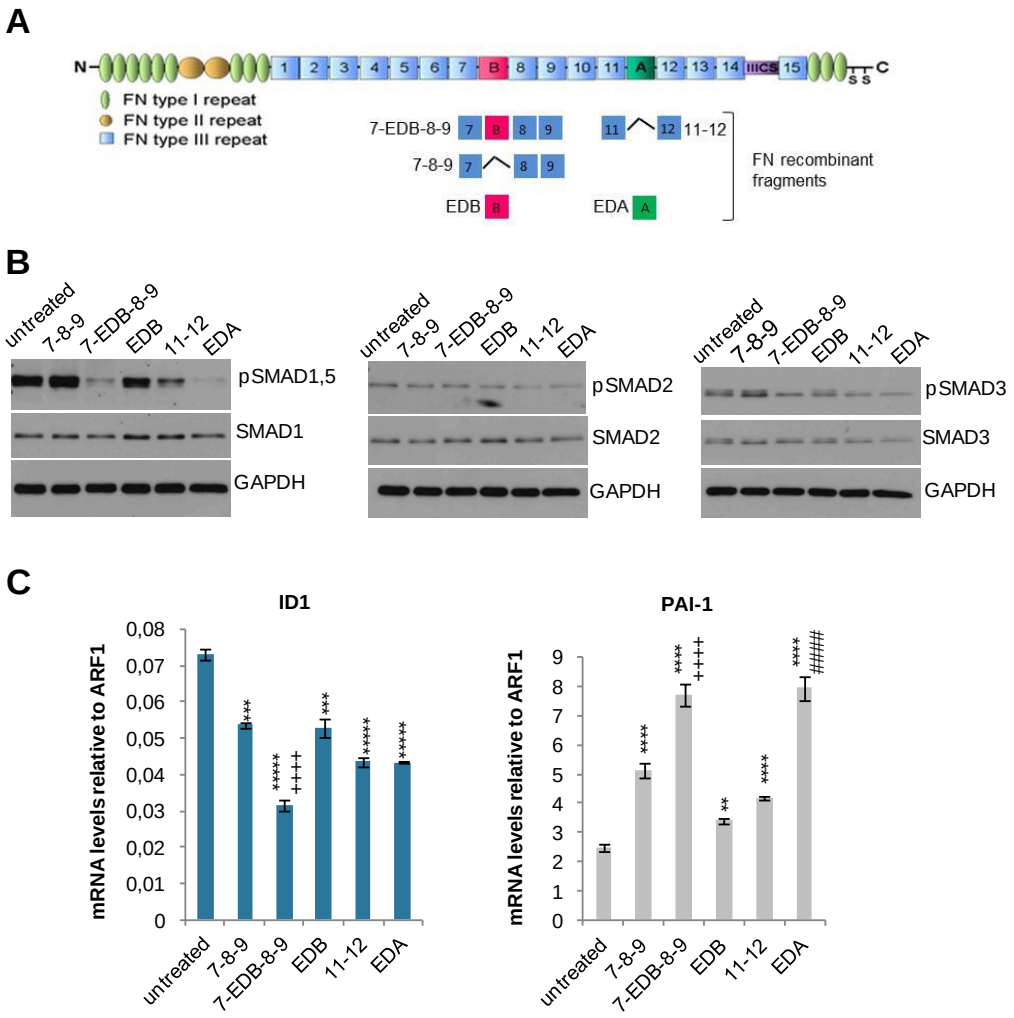

Figure 33. Treatment of hCMEC with recombinant fibronectin fragments containing the EDA and EDB domains reduces pSMAD1,5 levels. A. Recombinant fragments of the FN molecule adjacent to the EDA and EDB domains including and excluding the extra-domains A and B used in this study. **B.** HCMEC cells were treated with the indicated FN recombinant fragments at 1 mM for 48 h. Levels of phosphorylated and total SMAD1,5, SMAD2 and SMAD3 were determined in cell lysates by immunoblot. **C.** HCMEC cells were treated for 24 h with the indicated recombinant FN fragments. ID-1 and PAI-1 mRNA levels were determined by RT-PCR. Data are expressed as mean ± SD (***P*<.01, ****P*<.001, *****P*<.0001 treated *vs* untreated cells, ++++*P*<.0001 cells treated with 7-EDB-8-9 *vs* cells treated with 7-8-9 or EDB, one-way Anova followed by Tukey's post hoc test 95% CI; #### *P*<.0001 cells treated with EDA *vs* cells treated with 11-12, unpaired Student's t-test).

6.2.2.3 FN modulates α-SMA expression in endothelial cells derived from glioblastoma.

Since it has been shown that endothelial cells undergo EndMT and express α-SMA in glioblastoma (Huang *et al.*, 2016) and that EDA+FN controls α-SMA expression in fibroblasts (Serini *et al.*, 1998), we asked if EDA+FN or EDB+FN modulate α-SMA expression in endothelial cells derived from glioblastoma. To this aim we performed the selective gene silencing of EDA+FN or EDB+FN in four CD31+ cells lines derived from freshly dissociated glioblastoma tissue (ZH-464, ZH-483-2, ZH-613, ZH-616). In all the cell lines, EDA+FN and EDB+FN gene silencing reduced α-SMA mRNA expression (Figure 34), suggesting a role for EDA+FN and EDB+FN in the regulation of α-SMA levels in glioblastoma-derived endothelial cells.

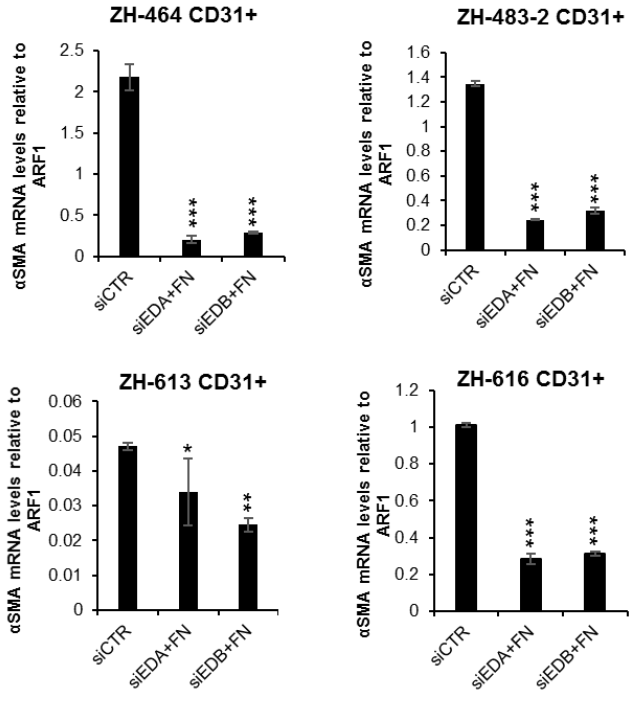

Figure 34. EDA+FN and EDB+FN control α-SMA expression in glioblastoma-derived CD31+ cells. α-SMA mRNA levels were determined by RT-PCR in the indicated CD31+ cell lines upon specific gene-silencing of EDA+FN, EDB+FN or non-targeting control. Data are expressed as mean ± SD (*P<.05, **P<.01, ***P<.001 unpaired Student's t-test).

6.2.2 Discussion

The fibronectin splice isoforms containing the EDA and EDB domains, EDA+FN and EDB+FN, are highly expressed in the vasculature and in the heart of the developing embryo (Astrof and Hynes, 2009). After completion of developmental processes, both EDA+FN and EDB+FN are down-regulated and are almost undetectable in mature adult blood vessels and tissues, with the exception of tissues undergoing physiological remodeling and angiogenesis, like the uterus and the ovary. By contrast, during pathological tissue remodeling, in chronically inflamed tissues and tumors, these FN isoforms are re-expressed and accumulate around newly formed vessels (Astrof and Hynes, 2009). In particular, EDB+FN is considered a marker of angiogenesis since its expression in the adult blood vasculature is strictly restricted to vessels undergoing neo-angiogenesis (Castellani *et al.*, 1994). Previous reports showed that FN is up-regulated in glioblastoma vasculature (Dieterich *et al.*, 2012) and that the percentage of vessels expressing EDB+FN correlates with tumor grade in gliomas (Castellani *et al.*, 2002). Here we show that the expression of FN and not only of the two isoforms EDB+FN, but also of the isoform EDA+FN is abundant and restricted to tumor vasculature in glioblastoma (Figure 27). In addition we show that CD31+ cells are the main source of FN in glioblastoma *ex vivo* (Figure 28).

Glioblastomas are characterized by high vascular density and vascular abnormalization with the typical formation of glomeruloid vascular structures (Wen and Kesari, 2008). TGF-β2 is highly expressed in glioblastoma and it has been attributed a central role in both tumor angiogenesis and vessels abnormalization. A direct role for TGF-β2 in regulating the phenotype of glioblastoma vessels, including the expression of components of the extracellular matrix, has been proposed. Indeed SMAD2/SMAD4 and SMAD3/SMAD4 complexes localize mainly to the vascular and peri-vascular areas in glioblastoma (Dieterich *et al.*, 2012). TGF-β induces FN in several cell types (Ignotz and Massague, 1986) and is one of the best characterized modulator of the alternative splicing affecting the EDA and EDB domains (Balza *et al.*, 1988; Borsi *et al.*, 1990; Viedt *et al.*, 1995). Here we show that TGF-β2 induces FN, EDA+FN and EDB+FN in hCMEC cells (Figure 29) and in CD31+ cells isolated from freshly dissociated human glioblastoma tissue (Figure 31). Induction of FN, EDA+FN and EDB+FN by TGF-β2 involves the SMAD-dependent/canonical TGF-β signaling pathway and specifically SMAD3. These data

suggest a role for TGF-β2/SMAD3/SMAD4 signaling in the control of the expression of FN and its splice isoforms EDA+FN and EDB+FN in glioblastoma vasculature.

In endothelial cells TGF-β may activate both the ALK-5/SMAD2,3-dependent and the ALK-1/SMAD1,5,8-dependent signaling pathways. The activation of the ALK-5/SMAD2,3-dependent signaling branch may promote ECM deposition and endothelium stabilization and maturation, whereas the ALK-1/SMAD1,5,8-dependent signaling branch may contribute to the activation of endothelial cells, promoting their migration and proliferation (Goumans *et al.*, 2002). It has been proposed that the equilibrium in the activation of these two signaling pathways finally determines the effect of TGF-β on endothelial state (Goumans *et al.*, 2002). Previous reports suggested that in human microvascular endothelial cells, FN and its receptor integrin α5β1 increase the ALK-1/SMAD1,5,8-dependent signaling branch, by promoting the formation of complexes of ALK-1 and the co-receptor endoglin (Tian *et al.*, 2012).
Despite the extensively documented expression of EDA+FN and EDB+FN in neo-vessels and in endothelial cells undergoing EndMT, the precise role played by EDA+FN and EDB+FN in vascular morphogenesis and plasticity is not yet clear (Astrof and Hynes, 2009). Double null mice for the EDA and EDB domains die at embryonic stage with severe vasculature defects (Astrof *et al.*, 2007). By contrast, single knockout mice for EDA and EDB containing FN isoforms are vital, show normal vasculogenesis (Fukuda *et al.*, 2002; Muro *et al.*, 2008) and normal physiological and tumor angiogenesis (Astrof *et al.*, 2004). In the present work we suggest a role for EDA+FN and EDB+FN in the modulation of TGF-β superfamily signaling in endothelial cells. Specifically we show that EDA+FN and EDB+FN affect TGF-β superfamily/SMAD1,5-dependent signaling by increasing the phosphorylation of SMAD1,5 in hCMEC (Figure 32-33).

Previous studies demonstrated that glioblastoma vessels express several markers of EndMT (Dieterich *et al.*, 2012) and that glioblastoma-derived endothelial cells display endothelial plasticity (Huang *et al.*, 2016). By undergoing the process of EndMT, endothelial cells acquire a fibroblast-like phenotype, become more invasive and drive the abnormal vascularization which is typical of glioblastoma. During this process glioblastoma-derived endothelial cells express mesenchymal markers,

including α-SMA (Huang *et al.*, 2016). α-SMA, the actin isoform typical of vascular smooth muscle cells and functionally associated with cell contractility, is one of the most important markers of EndMT. It has been reported that, by promoting TGF-β1 activation and α-SMA expression, EDA+FN favors the conversion of fibroblasts into myofibroblasts (Serini *et al.*, 1998; Muro *et al.*, 2008; White *et al.*, 2008). In the present work we confirmed that glioblastoma-derived endothelial cells express α-SMA and show that both EDA+FN and EDB+FN promote α-SMA expression in glioblastoma-derived endothelial cells (Figure 34). These data suggest that FN and the two isoforms EDA+FN and EDB+FN, which are up-regulated in glioblastoma vessels and are known markers of EndMT, may contribute to the EndMT process by promoting the expression of α-SMA in tumor-associated endothelial cells.

In summary, in the present work we show that TGF-β1 and TGF-β2 induce FN, EDA+FN and EDB+FN in hCMEC and in CD31+ cells derived from human glioblastoma tissues. In turn, EDA+FN and EDB+FN modulate TGF-β superfamily signaling by increasing the phosphorylation of SMAD1,5 in hCMEC and promote the expression of α-SMA in glioblastoma-derived endothelial cells.

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8. CURRICULUM VITAE

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Education

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Work experience

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Fellowships

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9. PUBLICATION LIST

- Ventura E., Weller M., Burghardt I**.** Extracellular signal-regulated kinase 1 (ERK1) mediates the autocrine positive feedback loop of TGF-β and furin in glioma-initiating cells. Accepted on April 14, 2017 for publication in *The Journal of Immunology.*

- Ventura E, Cordazzo C, Quarto R, Zardi L, Rosano C. C6: a monoclonal antibody specific for a fibronectin epitope situated at the interface between the oncofoetal extra-domain B and the repeat III8. *PLoS One* 11(2):e0148103, 2016.

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10. DECLARATION

Hiermit erkläre ich, dass die von mir vorgelegte und beigefügte Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen habe ich als solche gekennzeichnet.

Ich versichere außerdem, dass ich die beigefügte Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Zurich, 05/05/2017 Elisa Ventura

Ort, Datum Unterschrift